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CALLING ATTENTION TO 505

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ART AND ARTIFICE IN HISTOLOGIC SCIENCE*

Pío del Río-Hortega

Dr. Pío del Río-Hortega has for the past fifteen years been working "provisionally" in a little laboratory in the Residencia. With exemplary perseverance he has accomplished in that nook a work highly valued by investigators the world over, though perhaps not sufficiently so by ourselves. With original methods he succeeded in making even the most delicate details of cell structure visible. And through his work on the histology of the nervous system, he discovered a new cell species which the scientific world christened, in his honor, the "Hortega Cell." His research on tumors of the nervous system has made him an outstanding authority—a position confirmed by the latest International Cancer Congress. Dr. del Río-Hortega is a devotee of the plastic arts, and the article which follows is the fruit of the union of his artistic sensibility and his scientific activity. (Note by the Editor of *Residencia*).

Histology is an odd-tasting dish, repulsive as a medicament to students who must be examined in it, and little liked by physicians who consider their schooling finished. Taken in large quantities under compulsion it is not absorbed, but if tasted repeatedly in little sips it finally becomes a delight to

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*Arte y artificio de la ciencia histologica. In *Residencia, Revista de la Residencia de Estudiantes, Madrid* 4: 191–206, 1933. Translated by E. Winslow Wolfe, Boys High School, Brooklyn, New York, and Gertrude M. Butler, Army Medical Library, and adapted by Webb Haymaker, Army Institute of Pathology, Washington 25, D.C. This article owes its origin to the repeated urging of students of the University of Madrid that Hortega contribute something of his special liking to their bimonthly journal, *Residencia*. The particular issue in which it appeared was obtained by Dr. William Gibson in Madrid in 1936. Few if any other copies have reached this country and, even if they were numerous, the article merits translation into English, for it carries in rich, scintillating style, not without a touch of banter here and there, the philosophy of one of the scientific masters of our time. For an appreciation of Pío del Río-Hortega (1882–1945), see the note by Wilder Penfield (*Arch. Neurol. Psychiat.*, 54: 413–416, 1945), and the tributes by Miguel Prados and William C. Gibson (*J. Neurosurg.*, 3:275–284, 1946), and Moises Polak (*Arch. histol. norm. path.*, 3:377–421, 1947).

the palate and even a cause of addiction. He who has a sensitive perception of the artistic will readily appreciate the fact

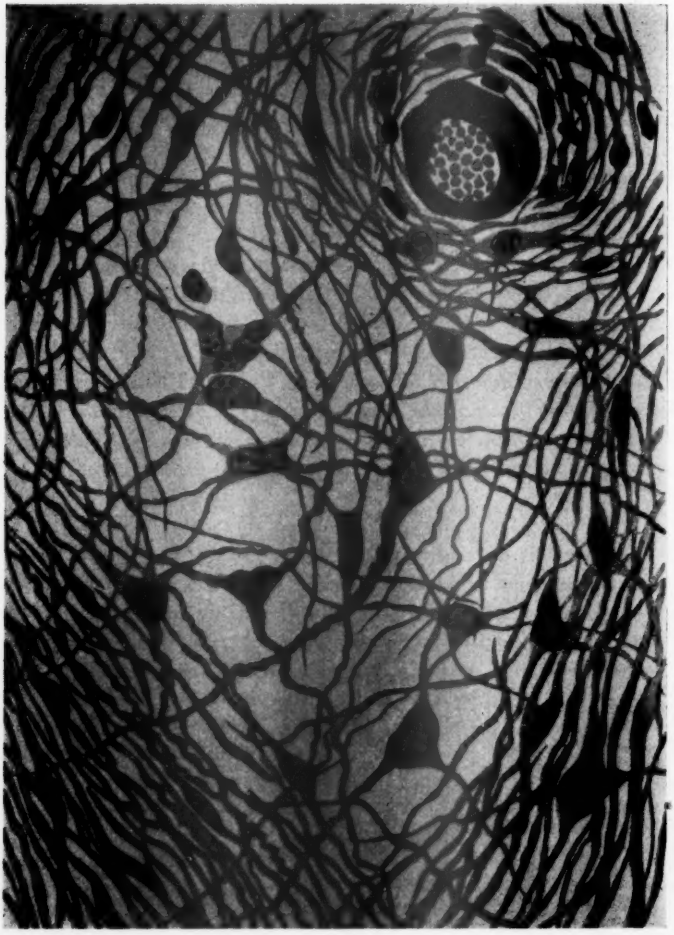

FIGURE 1

Even in the most malignant tumor, cells are grouped harmoniously, and in this tumor of the optic nerve they are star-like, with extremely long, intertwined arms.

that there exists in the science of histology a strong aesthetic appeal.

Those who make it their profession are content to earn little money and have, consequently, few enemies.

It is a respectable and a respected science which serves, among other more substantial things, to lend distinction to the other branches of medicine. In all good publications histology

FIGURE 2

The nerve cells are interrelated by means of protoplasmic ramifications (dendrites) and sometimes display graceful plumed forms.

holds a place of high esteem. At the banquet of the medical sciences it is a strange and mysterious guest of honor who eats little, to whom all give ear, and whom very few understand.

It is a pure and true science which is content with nothing less than the exploration and discovery of all the secrets of the fundamental architecture of beings, of organized matter, of the substratum upon which life rests.

When the histologist analyzes the structure of living tissues, he shreds and teases apart their elements in order to differentiate the cell morphologically, a process which epitomizes the general qualities of the individual and the special characteristics of the organ of which it is a part. But the cell, as an elemental organism—a living unit—encloses in its diminutive mass an aggregate of structures, or little organs, each of which plays an important physiologic role. The cytoplasm, with its tonic, trophic and kinetic structures (that is, those serving the functions of support, nutrition and movement), and the nucleus are, in the cell, equivalent to the nervous, digestive, urinary organs, etc., of the individual; and in the final analysis—for which microscopes are still useless—they are integrated by tiny living particles or cytobions which the metaphysically inclined scientists call gemmules, plastids, biophores, protomeres or pangens, analyzing them in their own special fashion for the purpose of determining and delimiting the concept of life.

Histology, then, in its purely scientific aspect becomes finally sublimated and merged with philosophy, although histologists are wont to replace the philosophic concepts, veritable imaginative creations, with precise and quintessential morphologic analyses.

So we find that the science which deals with the structure of cells and tissues has two aspects: one, rigid, static and given purely to meditation—or metaphysical; the other pleasing and dynamic—or artistic. I must confess that if our science had only of the first aspect, with its abstruse ideas, its chimeras and pedantic expressions, I should not be a histologist. I firmly believe that in order to be one, it is essential to have the soul of an artist and to be a true romanticist.

What does it matter to us, they may say to me, whether you define and practice the discipline you profess as a science or

as an art? What is of interest to us is that, after working for so many days in your laboratory, you should give birth to new truths, unveil many mysteries, extract from chaos extraordinary concepts, and that, since you are a biologist, you should solve the problems of life and of death—above all, those of that supreme good, health, which makes us love life, and of that supreme evil, sickness, which makes us fear death.

And such a viewpoint is a reasonable one. It cannot, however, be denied that a precise knowledge of the motives of those who consecrate their lives to a science is important for the present and the future of that science. I don't mean that it is important for everyone to know these motives, but it is essential for the scientists themselves (almost always unaware of the innermost source of their professional preferences and aptitudes) to do so and also for those who have drawn away from science without having tasted its attractive qualities.

Many shun science because they think it dry and barren. That is because they have accepted the myth of the heroism of those who practice it like a priesthood, the myth of the actruism of those who renounce more lucrative employment in order to satisfy their scientific enthusiasms in the service of country and humanity, and also the myth of genius, which would lead one to suppose that in order to make scientific discoveries it is necessary to have extraordinary understanding and intelligence. All these myths deserve rectification: science is not dry and insipid, and scientists do not have to be heroes, altruists or geniuses. Sometimes it is enough that they be artists. In fact, the investigator in histology must be an artist in order to master technique, to observe results, and to render a microscopic image faithfully.

Histologic craftsmanship, which means technique, must not be confused with aesthetic results. The compatibility of technical skill and aesthetics depends on whether one is dealing with the fine arts, especially the plastic arts, or with what might be called the fine sciences: the biologic sciences. In true art, pure art, technique is exclusively at the service of aesthetics, whether in support of or at variance with actuality. The artist seeks to create a beautiful work without considering the processes: altering, deforming, masking reality does not perturb him in

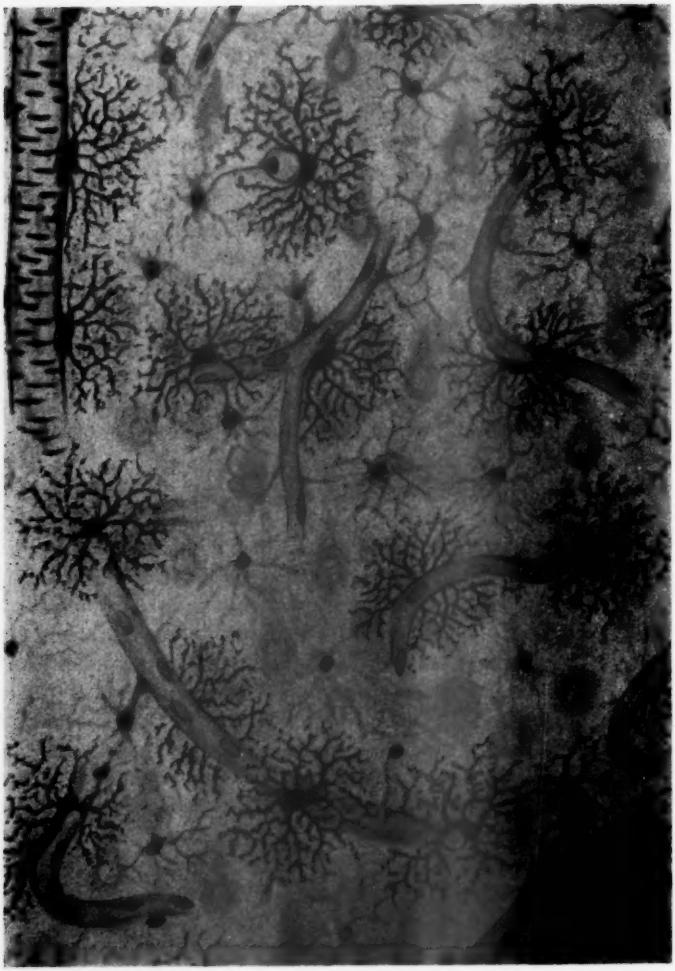

FIGURE 3

In the landscape of the cerebral tissue there are innumerable irrigation canals (blood vessels) and on their banks are thicket-like cells (neuroglia) which collaborate in nerve function.

the least if he succeeds in giving his creations aesthetic qualities, both in what is external and formal and what is internal and of most essential worth—and by these means, whether we wish him to, or not, he will penetrate our spirit and produce a surge of emotion.

The modern artist does not wish to portray forms naturalistically, but prefers to render only just enough of them to suggest a full representation. For this reason, modern painting and sculpture are based on the idea of surrealism or, rather,

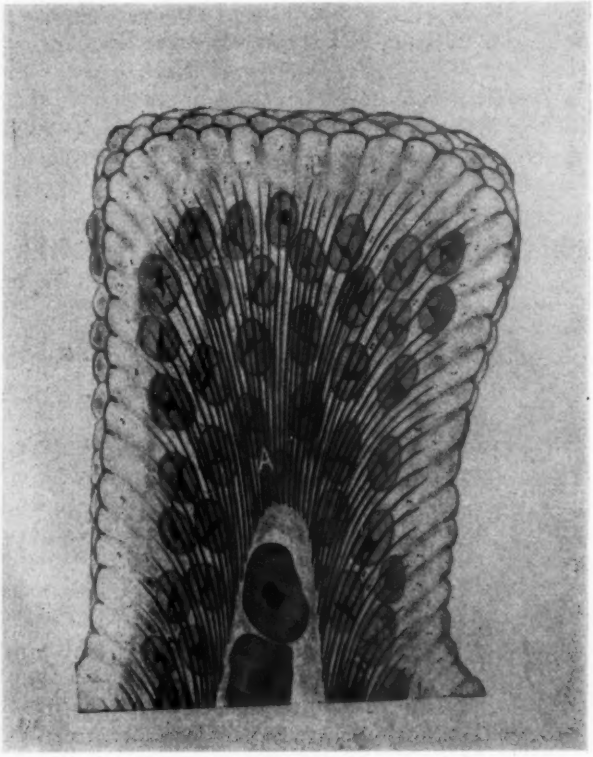

FIGURE 4

Neither the ugliness of the toad nor the stench of his intestine can be suspected in the fan formed by its epithelial cells.

unreality. In this connection, there is an ascending line of expression which, though beset with many pitfalls, climbs from the concrete to the abstract in pursuit of a stylization which brings out the peculiar qualities of objects by depriving them of every unessential detail—by suppressing actuality and magnifying aesthetic values. The ultimate is made possible only by a purification of technique.

In histologic science, technique is the faithful servant not of aesthetics but of actuality. It therefore shuns unreality, which is often hidden in artifact. The aspiration of the histologist is to come upon the perfect truth, the nature of which no one knows; in seeking it he makes surrealist sketches of what he sees (that is, figures that suggest forms which have no linear profiles), and on such a basis he fashions interpretations and constructs hypotheses, which make his science one of continual unrest, and at times it happens that the hypothesis is so well harmonized with facts, and so beautifully explains everything, that it may establish itself as nothing less than the truth.

This can also happen with the hypotheses that are developed in art.

Technical skill and aestheticism are two different routes which converge only in the supreme artist. Some triumph through technical dexterity and others through aestheticism, and it is possible to be a consummate technician, a virtuoso of technique, without achieving a beautiful work.

In the mother-lode of art, where everyone is eager to extract the glittering gold of originality, to find unheard-of forms of expression, it is possible to triumph either by technical skill or aesthetic creation. Picasso, for example, with his ingenuity, triumphs through the aesthetic values of his pictorial creations. Solana, on the other hand, goyanesque Solana, triumphs through technical ability. His works have no aesthetic substance; they are repulsive because of their tragic surrealism, which serves only to frighten away the spectator.

Among the painters of the avant-garde there are true creators of hypotheses comparable to those which are constructed in science by amalgamating fact and imagination. As we stand before many paintings there arises in our minds the question, "What can this be?" For in them we find, often in wisely

harmonized colors, unsubstantial forms mercilessly deprived of every vestige of reality. And we endeavor to answer the question in a thousand different ways. It happens occasionally that, with perseverance, we are able to interpret the occult

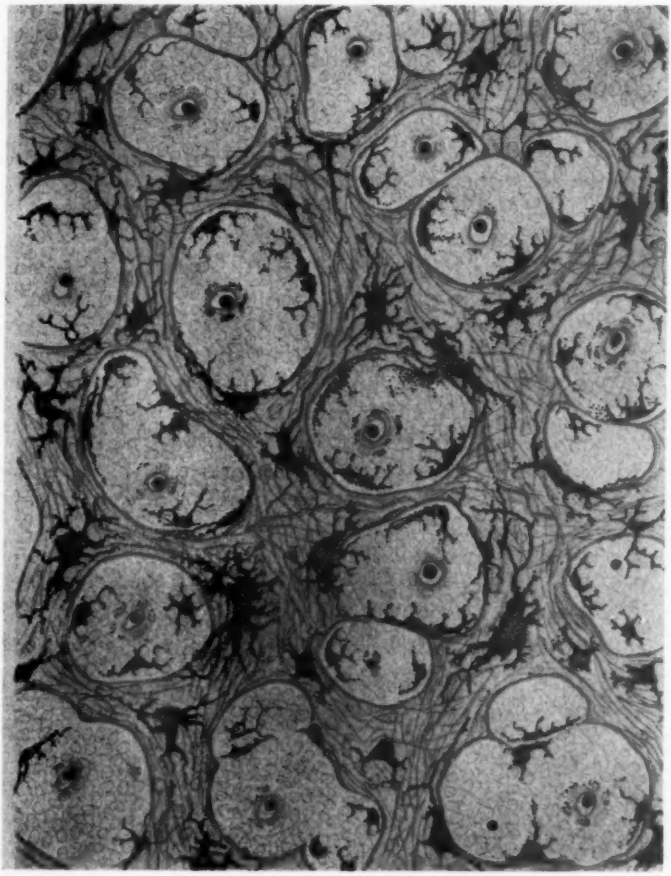

FIGURE 5

In the rabbit's skin there are black cells (melanophores) which give it color, and sectioned hairs that resemble open eyes in the orbit of their follicles.

meaning of what attracts us and we thereupon suffer a great disappointment. As the mystery is shattered and the hypothesis disappears, everything good that existed in the work, namely, emotion reflected in our curiosity and anxiety, fades away. For this reason, in many daring pictures of the avant-garde it is better to renounce analysis and be content with an hypothesis, if it is presented to us aesthetically and absorbs our interest.

I have a conception of science—at least of objective science—that is a little unusual. It may be thought that science is produced when one describes in minute detail what is before his eyes, which may indeed be of the greatest novelty and of transcendent importance. But, actually, science is produced when what is seen is interpreted and the essence of the phenomena is scrutinized. A pure description belongs to scientific literature and has only an external and episodic value. A hypothetic interpretation penetrates intimately into the facts and seeks the mystery of their existence. Anyone can observe what he has in front of him, but to interpret it intelligently, to hit upon a wise explanation, is within the reach of very few. Much the same is true in art. No longer does it suffice to describe minutely with brushes or modeling tools what one has in plain sight; it is necessary to interpret it, and then to give the interpretation substance. The artist, like the scientist, must seek the soul of things, which, of course, is superior to the plastic reality.

It is becoming apparent that there is a point at which art and science converge. If the particular science happens to be histology, the points of coincidence are even greater, for the study of the intimate contexture of the organs is founded on a technique which necessitates the use of color—one or many colors—whose qualities are wisely utilized.

To make visible the different elements which are associated in organic tissues and which form the living matter of cells is a task not to be accomplished without some difficulty and often constitutes an extremely complex problem. For that reason the so-called histologic technique, which might be defined here as the aggregate of artifices that lead to the revelation of histologic art, daily becomes more extensive.

In histology, as in the fine arts, those who have shone, and will always shine, are the creators of technique, who, with their aversion to following the beaten path and with their bent for innovation, have sought untried ways of expressing beauty or of clarifying truth.

FIGURE 6

The nerves of the leech have large axial cells with prolongations that radiate in all directions, giving support to the nerve fillets.

Just as in painting and in sculpture each period is manifested by the style or the interpretative manner of an extraordinary artist, which is expertly copied or bunglingly imitated by others more or less ordinary, so also in histologic science each important discovery corresponds to the invention of a fertilizing technique. The prodigious work of Cajal, his countless discoveries, are the facile result of his genius as a creator of techniques.

One can be a dilettante of music, painting or sculpture without knowing how to play an instrument, without ever having painted or modeled; but one cannot be a virtuoso of histology without at the same time being a performer. For this reason, those who do no technical work in the laboratory, but turn it over to routine assistants possessing hands and medulla but no brains, never can become expert histologists. Those who invent no techniques rarely succeed in making discoveries of importance.

Every still-unsolved biologic problem is in reality a technical problem and cannot be solved until an adequate technique is created. For those of us who in these days frequent the laboratories there is in comparison little to do, and each succeeding day even less; there remains only the little that was left undone by the first inventors and practitioners of histologic techniques and by those who after them exhausted everything their techniques were capable of yielding.

As when one surveys a panorama from the top of a hill, one can in the initial stages of an objective science make important discoveries with a minimum of effort. But each time one climbs the hill again it becomes necessary to look more intensively if one wishes to discover new truths and thus feel amply rewarded for the effort expended.

At first, it was enough to make use of elementary resources: a rudimentary microscope and a few very simple methods of demonstration. By these instrumentalities were made the discoveries that today bear the names of the masters, who revealed their greatness not so much by making the findings which brought them fame, but by being able to interpret those findings with keenness of insight.

FIGURE 7

In the healthy brain the nerve cells have bodyguards (Hortega cells) which extend their tentacles in every direction, and hold back whatever might be noxious.

Today we professional histologists need powerful microscopes and very complex techniques in order to glean much less valuable discoveries. For this reason an art of practicing histology by means of thousands of formulas and variations has been created. Its purpose is to give qualities of visibility to cellular structures, since in their natural state they are invisible because of their lack of color and their great transparency.

The complexity of the fabric in most tissues requires the use of staining techniques, either to show up the whole mass and forfeit precision of details, or to make plain the details at the expense of the whole. The use of these techniques results in the attainment of true histologic pictures which are a source of pleasure for the histologist who is sensitive to the grace and rhythm of the tectonic composition, to the beauty of the cellular forms, and to the harmony and transparency of the colors.

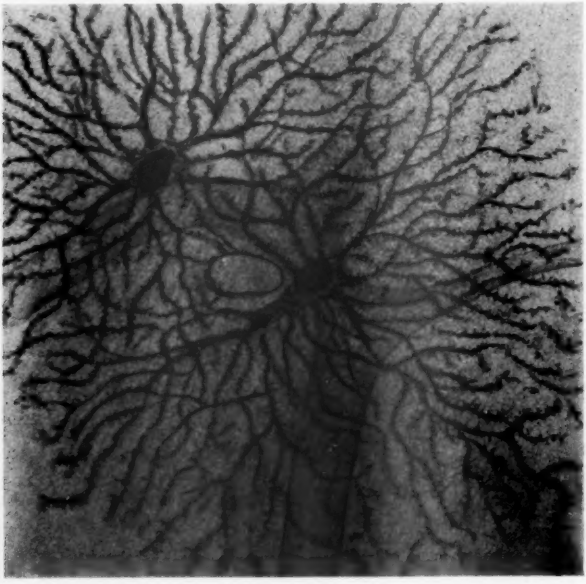

FIGURE 8

Without the luxuriant fern-like arborization of these cells (neuroglia) which envelops and isolates the neurons, the latter could not exercise their important functions.

The histologist's inventive faculty is taxed looking for new ways to take advantage of the chromatic appetites of each element or structural detail of the tissues and organs, since the difference in chemical composition of the cells and intercellular substances causes the apparent miracle that, from a mixture of various colors, each element selects, fixes and retains only one of those colors and always the same one. This results in polychrome figures in which the nuclei, for example, take red, with a subtle gamut of tonalities; the cytoplasms, yellow and orange; and the interstitial fibrous tissues, green and blue. Sometimes the colors are used separatively and successively, but the result is the same: the nuclei crave the basic stains, the cytoplasms the acid stains, and the various other structures whatever stain suits their own particular microchemistry.

By means of these devices, the histologist is enabled to discern not only the beauty of the cellular forms and aggregates, but also their chemical composition, from which he can deduce, after a study of these and other properties, the normal or the altered function of the cells. That is why all histologists wrack their brains thinking up original methods for revealing things which are already known, and also for discovering, with the aid of good fortune, new truths.

The happy discovery of a fruitful formula of histologic coloration is a cause for satisfaction and rejoicing, not only to the one who has happened upon it—more often by accident than by special research—but also to many other investigators, who see in every new formula two possibilities. The first is to exploit the formula, applying it to various investigations, with the certainty that there is always some profit, the profit being occasionally far greater than the capital of intelligence invested; the second, to modify the formula, reform it, by the process of preserving everything essential and of altering, ever so slightly, what is accessory.

By this convenient system, which, because it is not patented, is exploited profitably in all countries at the same time, one may succeed in acquiring fame as a clever technician, capable of finding and immediately eliminating the defects of someone else's technique and at the same time even changing its nationality. Thus, what was thought out in one country may

quickly acquire naturalization papers in another. A method devised, say, by Jones is published and cited as Smith's modification of Jones's method; but pretty soon Jones, being a foreigner, is ignored and the method becomes that of Smith.

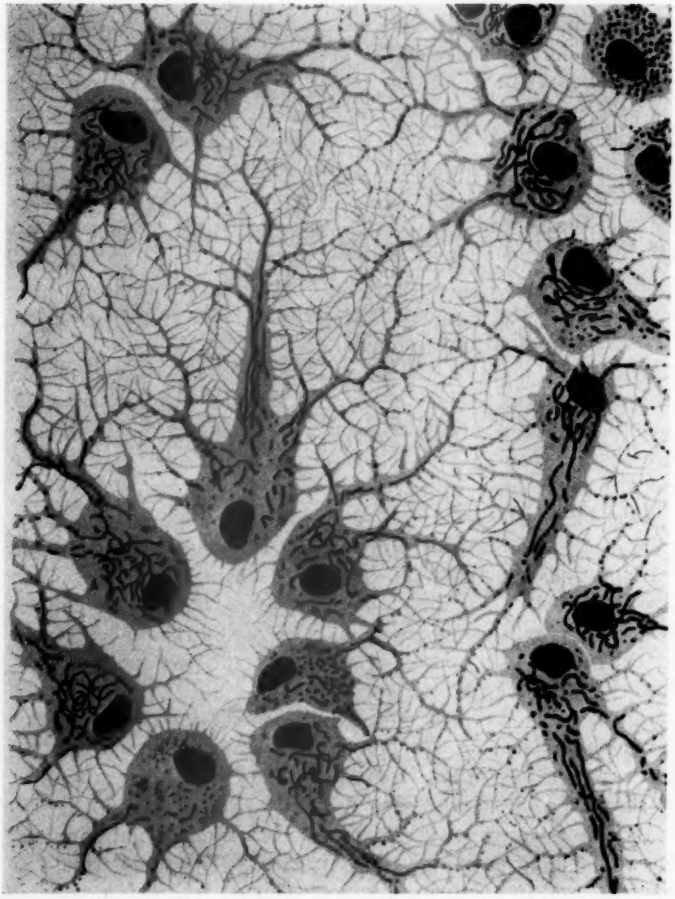

FIGURE 9

In the cephalic cartilage of the squid, the cells lead a life of repose and gather together as a family to exchange confidences.

In each country it is customary to give preference to a certain technical modality, which inevitably leads to revelations of a distinctive character. Because of this, scientific truth may not be the same in all countries, and there may even exist truths for every taste. What, in one place, constitutes the most impressive expression of a perfect technique in another place is despised as an artifact, which deforms and disfigures

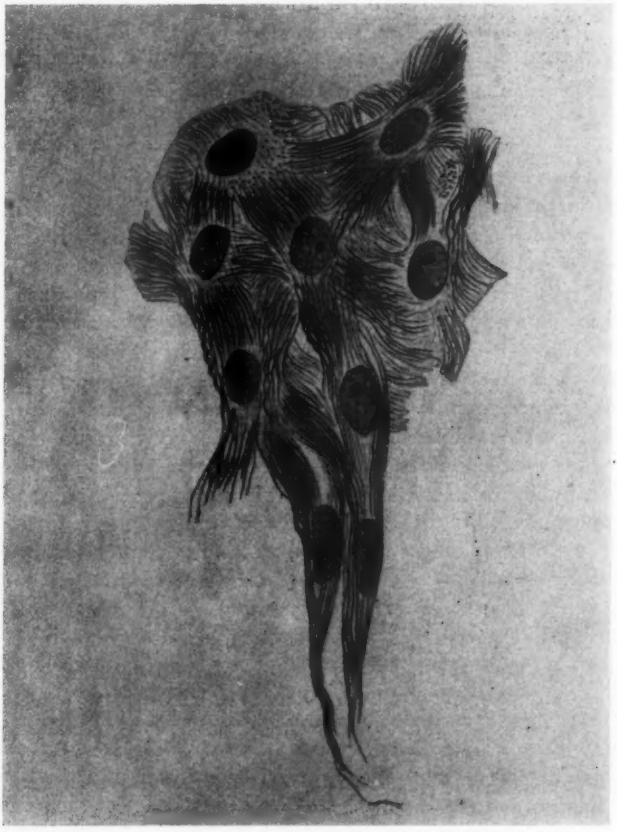

FIGURE 10

The cohesion and elasticity of the epidermis would not exist without the flexible threads that bind its cells.

reality. And thus it happens that what many consider the absolute truth others hold to be a relative truth, and still others, pure fiction.

The age of internationalization of scientific work is still a long way off, and it does not seem that scientists are very eager for it to arrive. The day must come, nevertheless, when investigators work in anonymity, applying themselves wholly to scientific progress, and are solicitously attended by the governing powers, who have become convinced at last that the sciences and arts set the true aristocratic standard for nations.

The present remuneration of the pure histologist is usually precarious and hardly sufficient to pay for the necessities of the style of living which is in keeping with his social position. Therefore he needs and looks for other types of reward. If he is to labor in obscurity for country and humanity, the inner satisfaction of duty fulfilled alone will not satisfy him. In order to persevere in his task he requires one or both of the following stimuli: the spur of public recognition (that is, present fame and future glory) and personal satisfaction in the work itself.

If public recognition is his incentive, the histologist looks upon science merely as a means for reaching the goal of his ambitions. Aided by plenty of well-trained subordinates, he becomes a wholesale manufacturer of science, looking for no pleasure in the process. If, on the contrary, personal satisfaction is his spur, the histologist regards science as an end in itelf and exhibits no ambition other than that of rejoicing in his discoveries. He works silently, delighting in his own labor. He makes a true art of his profession; and, like the painter who himself prepares his own canvases and pigments, he derives pleasure from the practice of various research methods, and gets excited over the results. The truths of science and the beauties of art are so amalgamated in histology that it is impossible to know whether the histologist has a passion for science or for its adornments: for the beauty of truth, or for the truth of beauty.

As for myself, I confess that I do not know whether a fervent eagerness to see what no one else has ever seen has made me a histologist—though the effort to do so has almost always

FIGURE 11

In a lesion of the brain (encephalitis), Hortega cells resemble voracious monsters and are valuable assistants in cleaning the tissue of whatever has damaged the nerve cells.

been fruitless—or whether it was an indefatigable urge to observe the structural marvels of tissues by cunningly tearing their veils away.

When, after using one of those technical devices in which it is necessary to combine meticulously varied complementary colors—red and green, yellow and blue—the histologist obtains a true pictorial image, he can find in the experience three clear wellsprings of purest emotion: that which arises from the beauty of the scene itself, with its polychromy, its modulation and its individuality; that which gushes forth from the observer himself, who feels an inner satisfaction of having achieved his purpose; and that which emerges from the newness of the facts laid bare.

The emotion aroused by any discovery, however small, magnificently rewards the histologist for the patient work he has performed in order to accomplish it. To wrest, by cunning strategems of technique, a secret from nature, to penetrate to its hidden meaning, to create pure science while delighting in aesthetic contemplation is, for the histologist, something which might be compared with eagerly desired paternity. The investigator cherishes his histologic discoveries as the children of his spirit, whom he considers his own creations, incorporeal children who invest their proud progenitor with the halo of a semigod and provide him with auguries of immortality.

In his admirable volume, *Reglas y consejos para la investigación biológica*, Cajal maintains that the indispensable qualities of the investigator are mental independence, a taste for science, perseverance in his work, devotion to his country and satisfaction with recognition only. He must also possess an artistic temperament which will lead him to contemplate the proportion, the harmony, and the beauty of things. The venerable master analyzes the foregoing qualities point by point, but he doesn't go beyond mentioning the one regarding an artistic inclination, which, in my judgment, constitutes a most essential quality in the histologist.

Cajal himself, in whom all the qualities essential to the investigator culminate, possesses an exquisite artistic sense, which manifests itself and sparkles in all his works not only through his delight in descriptions, in which he emphasizes the

elegance and beauty of things, but also through the matchless grace of his histologic drawings.

One who derives no pleasure from exploring a complicated structure and takes no delight in the perception of each one

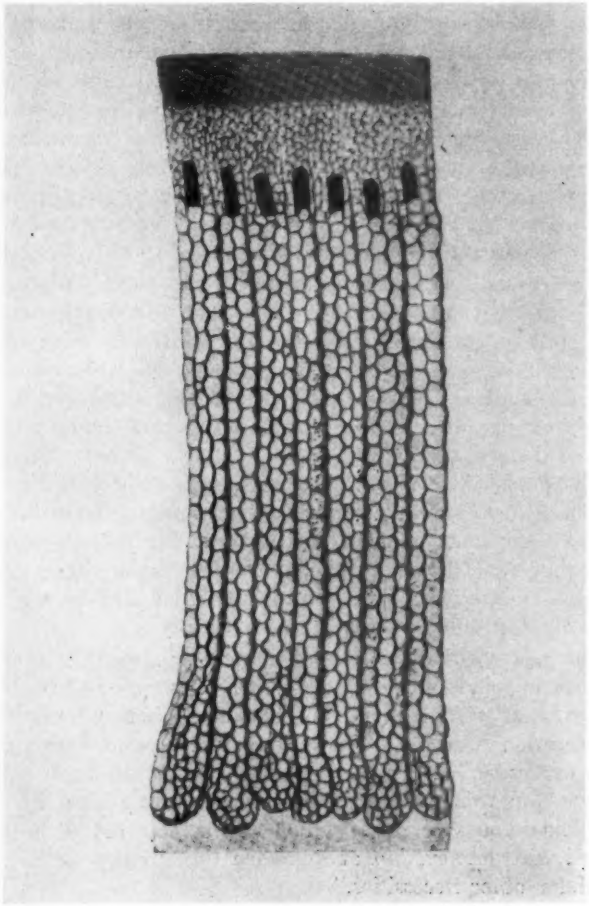

FIGURE 12

The cytoplasm of these branchial cells of a worm has cavities that give it a honeycomb appearance.

of its details will probably never find anything that has not been seen by others of greater sensibility and perspicacity. He who observes more, sees more; but only he who makes of histologic science an entertaining game, a recreation for the eyes and for the spirit, looks again and again without fatigue.

The artistic sensibility of the born histologist is such that he is moved by the delicacy of contours and the complicated architecture of the woven fabrics in which cells live both in a free state and in intimate union. He need not become excited by the glimpse of unsuspected truths or by the verification of controvertible ideas in order to have his feelings soar like a triumphal arch; that an image should appear before his eyes with greater beauty than in the schematic reproductions to be found in books and monographs is enough to satisfy him.

That person has not the makings of a histologist who, after being stirred emotionally before a perfect microscopic picture, does not look for someone of a similar sensibility with whom to share it, like a child gleefully showing his new toy. For with his smiling and optimistic spirit, the histologist of artistic bent has all the appearances of a child, living a little beyond the realities of life.

I have seen youthful enthusiasts of histology pass through my laboratory who lacked only the romantic spirit to become good investigators. Hardly initiated into the inner secrets of cells, they wished to apply their knowledge by setting up commercial laboratories, by becoming professors, or by training themselves in some more utilitarian specialty.

Young men either without orientation or already committed in favor of some clinical specialty, have also passed before my eyes who, after some months of histologic practice, found their real vocation in histology. Invariably, when one of these youngsters expressed his decision to enlist in the histologic legion, I gave him this warning: "Remember, young man, in this profession you will readily find satisfactions not to be purchased with money, but at the same time money is not provided for other necessities."

Young histologists are wont to consider themselves, for a time, well repaid by their feeling of satisfaction, but when they come face to face with reality and apprehend that one

does not live on ideals alone, it is then difficult for them to take another road.

Not all those who make a profession of histology draw near to it as to the sacred fire that engenders in them artistic con-

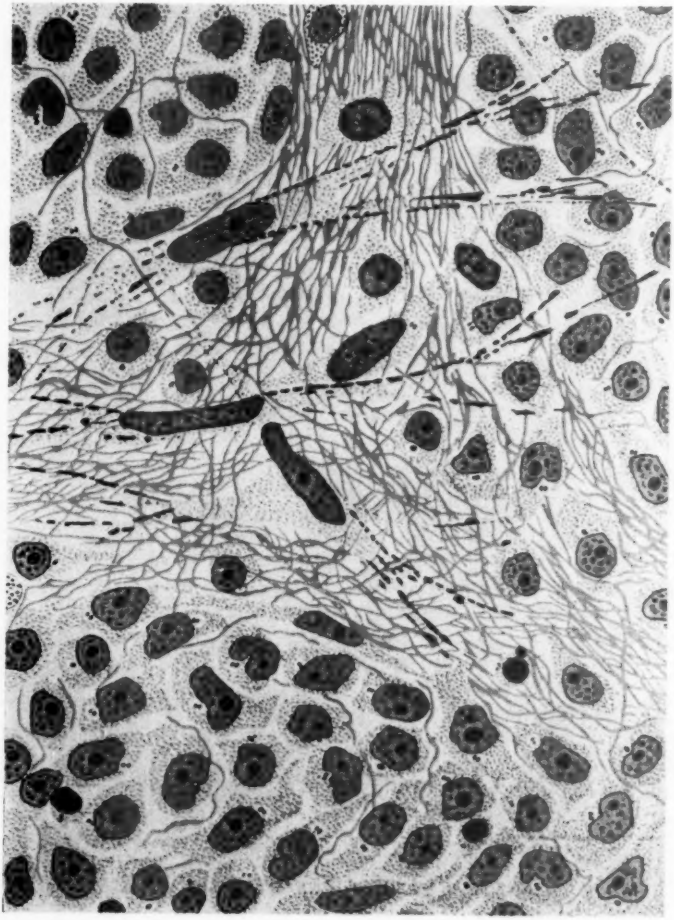

FIGURE 13

The frightful reality of cancer is masked in the beauty of its histologic imagery.

cepts. There are many to whom art means nothing and they seek in science only what serves their purposes. For this reason there are two types of men in the laboratories: Those, who, frenzied by their extreme speed, disregard everything, closing their eyes so as not to be distracted by the landscape, keeping only their goal in mind, and those who, in no hurry and each

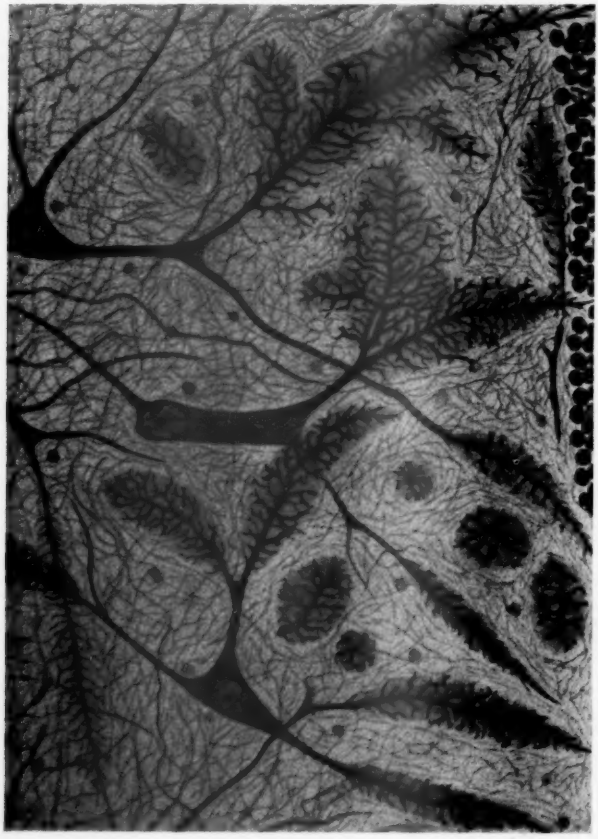

FIGURE 14

In the exhausted nerve cells of the senile dog, the protoplasmic arms have new shoots and look like flowering shrubs.

day doing their accustomed stint, allow their eyes free range so that they will miss none of the beauty of the roadside.

The first are the pseudo-scientists, who write precipitately about things half-seen and half-understood, confusing everything and bringing down upon their profession the horror of neologisms and of arbitrary hypotheses. To their number belong those who accept or reject on impulse the discoveries made by more serious workers after patient and laborious research and who concoct many theories which serve only to stir up contrary theories and to perpetuate controversies. They are convinced that in the acquisition of scientific prominence being wrong is worth just as much as being right and that fame comes to him who writes and speaks a great deal even if he invents or discovers nothing. They are the ones who for self-profit exploit the talent of others. I know some in this category, boasting the imposing title of professor, for whom gullible scientists—there are many professors among them—and the ignorant general public are full of admiration.

The others are genuine explorers of science who, seated at the side of the road, observe attentively, silently admiring the natural wonders, and who, lying in ambush for new truths, never fire a shot without hitting the mark. That is how the genuine craftsmen in histology conduct themselves. These investigators, who aspire, like all others, to live on in imperishable works, are likely to attain their goal; and, in the long run, not only do they obtain the rewards and honors of fame, but their names go down in history attached to some transcendent discovery.

But even though fortune should not prove propitious—sometimes because these men have not succeeded in devising fruitful techniques, sometimes because the priority of their findings is disputed or denied—they will, if they have cultivated science for their own pleasure, consider themselves repaid by the emotional satisfaction which the pure art has given them. Time is never lost in histologic investigation, for even if nothing new is revealed, beautiful designs are achieved and if there was difficulty in obtaining them, there is all the more satisfaction of accomplishment.

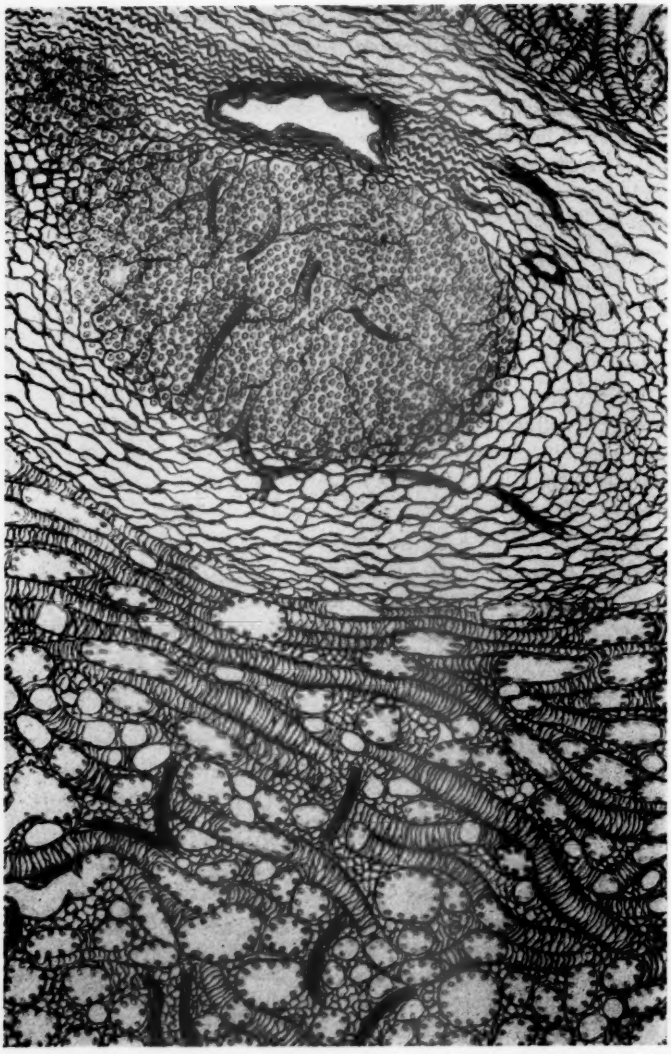

FIGURE 15

In the spleen there is a delicate grill-work of supporting and uniting fibres (connective tissue fibres) which serve as the weft of the tissue.

There are scientific disciplines which hardly ever change in their outlook, and which offer a broad, but monotonous, view; there are others that, by requiring the handling of foul-smelling and noxious gases or of virulent germs, demand a certain amount of abnegation. Histology lacks these dangerous features—it is practiced without any risk whatsoever—and offers the incentive of infinite variety.

The organs and tissues are always the same and always different. Identical in their fundamental architecture, they vary infinitely in details and, in them, no image is ever repeated The same is true of the cells. In each tissue they are different in form—round, polyhedral or star-shaped—and different in grouping, the varieties of each morphologic type being innumerable. But this is not all: the tiny organs of which the cell is composed offer anatomic, physiologic, and pathologic changes which multiply their images anew. The contemplation of them is a joy and a spur for the intellect, which labors without pause to discern the meaning of these variations.

It is not possible to talk about structures without having them in view and, consequently, our studies must of necessity be abundantly illustrated. Outside of Spain, investigators themselves often stammer out vague descriptions, but more frequently turn over the interpretation of microscopic preparations to well-paid professional illustrators. This error, committed in many centers of scientific production, is a gross one, for histologic data can be well perceived only by the investigator himself who, if he is not clever in reproducing them faithfully and gracefully, will give them, in lieu of this, an exact theoretic interpretation.

In Spain, following the magnificent example of Cajal, that great master of histologists, the traditional way is for each histologist to prepare his own drawings. Cajal's drawings are distinguished by their delicacy of outline in cellular silhouettes, their adroit superposition of strata, and their wise synthetic composition when it is necessary to assemble in a single drawing the elements apprehended in many microscopic fields.

I wish to insist upon the thesis that histologic science seduces the novice through the aesthetic stimulation that he finds in it

and that it rewards him generously for his efforts to discover its multiple beauties.

An interest in pure science begins with the establishment of the first hypothesis and is clinched by the first discovery. The latter arouses and magnifies dormant aspirations, so that the feeling for art and the passion for science become fused with a noble longing for immortal fame.

The investigator's spirit burns, without being consumed, in a lamp of three flames: the many-colored flame of artistic sensibility; the bright white flame of eagerness to discover new truths; and the blue flame, whose vortex soars upward and seeks eternity.

ENTERIC PATHOGENS ISOLATED FROM CHILDREN IN SAN ANTONIO*

MARY L. BEENE, S. W. BOHLS, AND MACDONALD FULTON

This report of the enteric isolations made in the bacteriology laboratory of the Santa Rosa Hospital in San Antonio, Texas, covers a period from June, 1946, to June, 1948. With only a few exceptions these isolations were obtained from infants and young children from the Latin-American population.

In order to detect carriers and obtain cultures as early as possible, a rectal swab was taken on each pediatric admission before medication was begun. A second specimen was obtained the following morning. This procedure was followed irrespective of the admission diagnosis. If diarrhea was present daily specimens were obtained until the release of the patient. Rectal swabs were preferred to stool specimens because of the ease of obtaining the specimen and the convenience of examination. Stool examinations failed to give a greater yield of isolations than the rectal swabs.

When the rectal swab was taken it was placed in a tube containing 2 ml. of a preservative broth. A Difco Experimental Medium was employed. When the swab was received in the laboratory for culturing, it was removed from the broth and used to inoculate one quarter of a Bacto-Eosin Methylene Blue plate and one-half of an SS plate. This inoculum was spread over the entire plate with a loop needle. Since Bacto-EMB is more differential than selective, even a small amount of inoculum could contain so many organisms that the colonies would not be sufficiently isolated. To insure well isolated colonies the loop with which the Bacto-EMB plate was streaked was used without reloading for the inoculation of a second plate of this medium. After the plates had been inoculated, the swab was placed in a tube of tetrathionate broth. If there was any preservative broth remaining in the tube this was added to the tetrathionate. The next day this enrichment medium was streaked on an SS plate.

*From the Santa Rosa Hospital, San Antonio, and Department of Pediatrics, The University of Texas School of Medicine. Received for publication May 12, 1949.

In some cases Bacto-EMB gave a better yield of Shigella, Eberthella, and some of the paracolon strains. EMB and SS were found to be equally good for the isolation of *Salmonella*. *Paracolon bethesda* and *Proteus rettgeri*. Tetrathionate broth definitely aided in the isolation of these species. Overnight incubation of the enrichment medium was found best. If the tetrathionate broth were incubated 48 hours or longer the subculture plates were likely to yield only coliforms, *Proteus* or *Pseudomonas*. Bismuth sulfite agar was used in the first six months of this study but was discontinued when it failed to increase the number of isolations. The number of typhoid cases was small and the other media in use were adequate for the isolation of the organisms encountered.

All suspicious colonies were stabbed and streaked on Triple Sugar Iron Agar (BBL) slants with generous butts. All noncoliform isolates were checked for their biochemical reactions and, if possible, a serological typing was made. Included in the biochemical studies were the fermentation of glucose, lactose, maltose, mannitol, sucrose and xylose. Ten per cent lactose slants and Chilton's broth (Chilton, 1946) containing four carbohydrates which are not fermented by the Salmonellas but are fermented by some of the paracolons were used to aid in the separation of these groups. The utilization of citrate and urea and the IMVIC reactions were determined. A semisolid medium, Bacto-Motility Medium, was used to determine motility, as the hanging drop method did not prove reliable.

The chart shows the five biochemical characteristics which were most important in the differentiation of the isolates in this enteric study. Indol, which was important for complete identification, was not included in this chart since the separation of the groups presented was not dependent on this property. The Salmonellas and *Paracolon bethesda*, which both produce hydrogen sulfide, could be differentiated by the slow fermentation of 0.5% lactose broth by paracolon strains. By using 10% lactose slants a more rapid distinction could be made. These two groups of organisms could be separated from the Shigellas and *Proteus rettgeri* on the basis of the anaerogenic fermentation of glucose by the latter groups. The Shigellas and *Proteus rettgeri* may be differentiated on the basis

of the utilization of urea and the motility of the Rettger's bacillus.

CARDINAL CHARACTERISTICS OF CERTAIN ENTERIC PATHOGENS

	H_2S	Glucose	Lactose	Urea	Motility
Salmonella	+	AG	—	—	+
Paracolon bethesda	+	AG	+s	—	+
Shigella	—	A	—/+s	—	—
Proteus rettgeri	—	A	—	+	+

The Salmonella isolates were submitted to Dr. P. R. Edwards for typing.

During the two-year period Salmonellas were recovered from 96 individuals. Of this number 5 were *Salmonella typhi*. There were 21 different types of Salmonella encountered. *Salmonella typhimurium* and *Salmonella derby* were most frequently isolated. Other types identified were anatum, bareilly, copenhagen, give, kentucky, kunzendorf, london, montevideo, newport, oranienburg, oregon, paratyphi B, rubislaw, saint-paul, seftenberg and worthington. The primary identification of the Salmonellas was based on the appearance of the TSI slant. When a slant showed acid and gas in the base, an alkaline slant and hydrogen sulfide production the organism was checked for agglutination in a polyvalent or pooled Salmonella antiserum. The culture was checked for the typical biochemical reactions. The incidence of Salmonella isolations was highest during the latter part of the summer with the peak being reached in August.

Beginning in the spring and continuing until about midsummer the Shigella isolations predominated. Another rise in incidence could usually be expected in the early fall. The *Shigella paradysenteriae* types were most frequently isolated. A total of 68 isolations were made. Types II and III, which are the W and Z types of Andrewes and Inman, and type VI, the Newcastle bacillus or 88 of Boyd, were the most prevalent. Types I, IV, V, VIII and XIII were also identified. *Shigella sonnei* was obtained from 46 patients. *Shigella dispar*, *Shigella schmitz*, *Shigella alkalescens* and the newly-described species *Shigella tieté* (Weil and Slafkovsky, 1948) were also isolated.

The Shigellas were identified by the lack of gas production in the acid base of the TSI and an alkaline slant. Extreme care must be taken in reading for gas production as small bubbles along the line of stab may be mistaken for gas. Lederle antisera were used in typing Shigellas and the biochemical reactions were determined.

Since we were unable to isolate a Salmonella or Shigella from all cases of diarrhea we were interested in identifying other organisms which might be of significance. *Proteus rettgeri* was one of the organisms which attracted our attention. We were able to identify this organism by its fermentation of urea, the motility, indol production and the anaerogenic fermentations, to which attention was called by Fulton and Curtis (1946b). Later in our studies a serological typing was included in the identification. *Proteus rettgeri* was isolated from 15 patients, 12 of whom had diarrhea. Two of these cases were fatal and one was a chronic diarrhea case on whom the organism was repeatedly isolated.

Paracolon bethesda (Barnes and Cherry, 1946) was also of special interest because of its distinctive biochemical and antigenic characteristics. This organism produces hydrogen sulfide, gives slow fermentation of lactose, fails to produce indol and gives a negative reaction in Chilton's medium. Edwards *et al.* (1948) described eight serotypes in the Bethesda group. We recovered organisms falling into the Group 1:1 serotype from 32 cases. Some of these had diarrhea while others gave no history of a recent attack. The actual figures appeared to indicate the existence of a high carrier rate. This group is similar to the Salmonellas in that they may also occur extraintestinally. A *Paracolon bethesda* was recovered from the bile of a gallbladder that had been removed from a suspected typhoid carrier. Another isolation of this paracolon was made from a draining sinus in a chronic osteomyelitis case. In view of these cases and those reported in the literature it was felt that pathogenic significance might be attached to *Paracolon bethesda.*

We were also interested in members of the genus *Eberthella.* The organisms we have called *Eberthella* were classified on the basis of the characteristics described in the fifth edition of

Bergey's Manual. This genus is non-existent in the sixth edition of the Manual (Breed *et al.* 1948). Certain authorities now classify these organisms as anaerogenic paracolons. The number of Eberthellas isolated was approximately equal to those of *Proteus rettgeri.* Biochemically these organisms are closely related, as was noted earlier by Fulton and Curtis (1946b). In some instances we could not be certain of the allocation of the strains since they exhibited characteristics of both groups. The presence of Eberthellas in the stools of diarrheal patients in many cases appeared significant although normal individuals also harbored them.

The incidence of Proteus and Pseudomonas isolations was high. Although species identification was not complete in all instances Morgan's bacillus, *Proteus vulgaris* and *Proteus mirabilis* were all encountered. The *Pseudomonas* were largely *Pseudomonas aeruginosa.* It was difficult to know whether to attach significance to these organisms.

The non-lactose-fermenting enteric bacilli found in diarrhea and dysentery in Texas included considerable numbers of *Paracolobactrum, Proteus, Pseudomonas* and other genera in addition to the more usual *Salmonella* and *Shigella.* Bohls (1948) noted the occurrence of species from all these genera. The same roster of genera and species was observed by Fulton (1945) whose sources included more adults than was true in the present study. Watt (1947) made extensive studies of the incidence of shigellosis and also reported salmonellosis, including three new types of that genus. Fulton and Curtis (1946a) were the first to report the use of modern serological methods of typing *Shigella* in this region, and noted the frequent occurrence of the Newcastle bacillus, type VI.

It was our conclusion that the techniques employed were simple and could be followed by any laboratory with even a limited amount of equipment. By the use of two types of plating media and an enrichment medium the number of isolations was increased. All organisms which are non-coliforms should be studied biochemically and if possible serologically, especially when isolated from cases of diarrhea. Only a rough or group serological typing to identify the genus is necessary in the small laboratory. The recognized pathogens and those

organisms which may be of doubtful significance or uncertain classification should be submitted to a central laboratory for complete identification.

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ISOLATION OF THREE SALMONELLA TYPES FROM ONE CASE OF SALMONELLOSIS

DONALD G. WETHERBEE, DOROTHY T. ANDERSON, AND DWIGHT M. KUHNS*

The isolation of more than one type of Salmonella from the same individual has been reported on a number of occasions, but recovery of multiple types is nevertheless unusual. Hormaeche, Peluffo and Allepo (1) isolated *S. derby*, *S. newport*, and *S. montevideo* from a child affected with gastro-enteritis. This appears to be the only previously reported case where three Salmonella organisms were simultaneously implicated in man. Cernozubov, Filipovic and Stravel (2) have reported man. Cernozubov, Filipovic, and Stavel (2) have reported from the same individual. The same investigators have also found two instances in which *S. paratyphi B* and *S. typhi* were isolated from patients and one instance each in which *S. senftenberg*, *S. derby* and *S. cholera-suis* var. *kunzendorf*, respectively, were found in combination with *S. typhi*. Edwards and Bruner (3) have described multiple infections in fowls, and in 1946 Bruner and Joyce (4) reported nine instances of double infection, but none with more than two serologic types. Other writers have reported isolated cases of double infection.

This paper reports an additional case where three serologic types of Salmonella were isolated from the same individual.

Case Report

A 25-year-old medical officer was admitted to Brooke General Hospital on 1 September 1948 with nausea, vomiting, abdominal cramps and diarrhea of six hours' duration. A month previously he had been hospitalized in El Paso for diarrhea and vomiting, which subsided after three days without a specific etiologic diagnosis having been made. A year previously he had had a similar short-lived attack for which he had not been

*From the Fourth Army Area Medical Laboratory, Brooke Army Medical Center, Fort Sam Houston, Texas. Received for publication May 6, 1949.

hospitalized. His past history was otherwise unremarkable, except that his sister-in-law died a year previously of amebiasis and the patient has been in frequent contact with her two children, both of whom also have amebiasis.

On the day before admission the patient had indulged in unaccustomed severe physical exercise, during which he perspired profusely and following which he felt weak. On admission to the hospital, he was trembling and apparently acutely ill, and showed signs of obvious dehydration; otherwise the physical examination was unremarkable. Temperature was 102.4°, and the pulse was 120. Patient was vomiting frequently, and passing loose stools containing blood and pus cells. His white blood count was 12,500, with 94% polymorphonuclear leucocytes.

On admission, the patient was treated with saline-glucose infusions and with morphine. On the second hospital day he was given two doses of streptomycin by mouth, but the drug was discontinued because of the patient's vomiting. By the sixth hospital day he was asymptomatic, though weak. Because of the stools positive for Salmonella, streptomycin one gram orally three times daily, and succinylsulfathiazole two grams every four hours were given during the period September 13 to September 27. On September 20 the stool was negative for pathogens, but Salmonella reappeared two days after treatment was completed, and continued to be present in the patient's stools for about two months, although during this time he was asymptomatic.

Laboratory Studies

Three types* of Salmonella were recovered from the patient's stools during the present illness: *S. bareilly*, *S. oranienburg* and *S. montevideo*. These organisms have the antigenic components noted in Table I.

*These typings were confirmed by the Army Medical Department Research and Graduate School, and by Dr. P. R. Edwards of the U.S. Public Health Service Communicable Disease Center, Chamblee, Georgia.

TABLE I

Antigenic Formulae

	O Factors	*H Factors*
S. oranienburg	VI, VII	mt—
S. bareilly	VI, VII	y 1, 5
S. montevideo	VI, VII	gms—

Bruner (5) has pointed out that it is possible experimentally to transform *S. oranienburg* to *S. montevideo*, and it is interesting to speculate whether this could have occurred *in vivo*.

TABLE II

Date	*Source*	*Organism*
2 Sept 1948	Feces	*S. bareilly*
13 Sept 1948	Feces	*S. montevideo*
20 Sept 1948	Feces	No pathogens
29 Sept 1948	Feces	*S. oranienburg*
8 Oct 1948	Feces	*S. oranienburg*
11 Oct 1948	Feces	*S. oranienburg*
21 Oct 1948	Blood	No bacteria
8 Nov 1948	Feces	(1) *S. oranienburg* (2) *S. montevideo*
29 Nov 1948	Feces	*S. oranienburg*
27 Nov 1948	Feces	No pathogens
28 Dec 1948	Feces	No pathogens
29 Dec 1948	Feces	No pathogens
30 Dec 1948	Feces	No pathogens
31 Dec 1948	Feces	No pathogens
1 Jan 1949	Feces	No pathogens
25 Jan 1949	Feces	No pathogens

It will be noted in Table II that only on one occasion were two types recovered from the same specimen. On this occasion six separate colonies were picked and studied serologically. Of course, five were *S. oranienburg* and one, *S. montevideo*. It is probable that had previous specimens been studied in more detail, multiple types might have been isolated.

Repeated warm stool and proctoscopic examinations for *Endameba histolytica* were negative.

The question of the pathogenicity of these Salmonella strains may arise, in view of the patient's clinical recovery while still harboring the organisms. However, Morgan (6) points out that individuals recovering from Salmonella infections may continue to excrete the bacilli for from 4 to 16 weeks after their illness, and that these temporary convalescent carriers are the most important sources of infection. On the other hand, chronic carriers of Salmonella other than *S. typhosa* are unusual.

Table III summarizes the immune response of the patient, as measured by the development of agglutinins against the Salmonella isolated from his stools and against stock cultures of the same types.

TABLE III

Agglutination Reactions

Patient's* Serum	*S.* oranienburg (stock)	*S.* oranienburg (patient's)	*S.* bareilly (stock)	*S.* bareilly (patient's)	*S.* montevideo (stock)	*S.* montevideo (patient's)	*S.* typhosa "O"	*S.* typhosa "H"	*S.* paratyphi	*S.* schottmuelleri
8 Sept 48	0	80	0	40	0	40	80	160	40	80
9 Nov 48	0	0	0	40	0	0	320	640	160	640
27 Dec 48	0	20	0	0	0	20				
26 Jan 49	0	0	20	0	0	0	320	320	80	160

*Patient received triple typhoid vaccine on 1 Nov. 48.

Agglutinins for the causative organism can usually be demonstrated in the circulating blood of patients from one to two weeks after the onset of Salmonella gastro-enteritis of the more severe types. However, in mild diarrheas the agglutination reaction may in some instances remain negative (6).

Jager and Lamb (7), studying eight cases of sporadic infection with *S. suipestifer* and *S. oranienburg*, found that in six cases the patient's serum agglutinated the isolated organisms in dilutions ranging between 1:160 and 1:1280. Two of the patients failed to develop agglutinins. In our case the patient

had a titer of 1:40 against *S. bareilly*, 1:40 against *S. montevideo*, and 1:80 against *S. oranienburg*. However, these titers are of doubtful significance, since the titers dropped to lower levels or to negative on subsequent specimens.

Jager and Lamb (7) also found that their two strains of *S. oranienburg* were lethal when injected intraperitoneally into mice. All three of our strains failed to produce death when injected intraperitoneally into mice in dosages up to 9,000,000 organisms.

In vitro sensitivity studies were performed for each of the three Salmonella strains with penicillin, streptomycin, bacitracin, polymyxin, aureomycin and chloromycetin using the tube dilution method, FDA broth as a medium, and complete inhibition after 24 hours as the end-point.

TABLE IV

Antibiotic Sensitivity

(Tube dilution method—FDA broth)

Organism	Penicillin	Streptomycin	Polymyxin	Bacitracin	Aureomycin	Chloromycetin*
S. oranienburg	1 u	10 u	3.125 mcgm	250 u	50 mcgm	3–5 mcgm
S. bareilly	1 u	10 u	.78 mcgm	250 u	50 mcgm	3–5 mcgm
S. montevideo	1 u	10 u	.78 mcgm	250 u	50 mcgm	3–5 mcgm

On the basis of these tests (Table IV) it is possible that polymyxin or chloromycetin might have been clinically useful. Stansly, Shepherd, and White (8) found susceptible strains of gram-negative bacteria inhibited by concentrations of polymyxin between 0.5 and 16 micrograms per ml. On this basis our Salmonella types, two inhibited by 0.78 and one by 3.12 micrograms per ml., could be classified as sensitive. Blood levels between 30 and 100 mcgm/ml. are obtainable with dosages of 2–4 gm. of chloromycetin daily, and all three of our strains were inhibited by 3 to 5 mcgm/ml. During the time

the patient was harboring Salmonella, we did not have sufficient quantities of either of these antibiotics for a therapeutic trial.

Summary

1. A case in which three types of Salmonella *(S. oranienburg, S. bareilly* and *S. montevideo)* were isolated from the same individual during an episode of acute gastro-enteritis is reported. Salmonella persisted in the patient's stools for about two months following clinical recovery.

2. No significant agglutinins against these organisms appeared in the patient's serum. The organisms were not pathogenic for mice.

3. *In vitro* sensitivity studies indicate possible sensitivity of these strains to polymyxin and chloromycetin, but not to penicillin, streptomycin, bacitracin or aureomycin.

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*If the technique of 50% of growth (measured turbidimetrically) of control (Parke, Davis & Co. method) is used, considerably lower figures for sensitivity to chloromycetin are obtained.

SPRING MEETING, TEXAS BRANCH, SOCIETY OF AMERICAN BACTERIOLOGISTS

May 7, 1949

The University of Texas Medical Branch, Galveston

PROGRAM

Infant Diarrhea in San Antonio
Frank W. Chorpenning, 4th Army Area Medical Laboratory, Fort Sam Houston, Texas

Lunch time symposium on Recent Advances with Antibiotics and Chemotherapy—Medical College Cafeteria

Introduction—Chauncey D. Leake
Malarial Diseases—Wendell D. Gingrich
Mycotic Diseases—E. A. Johnson
Bacterial Diseases—C. E. Lankford
Rickettsial Diseases—L. Anigstein
Virus Diseases—S. E. Sulkin

Macrophage Promoting Factor in Rabbit Serum
C. M. Pomerat and M. F. Orr, The University of Texas Medical Branch, Galveston, Texas

Destruction of Nutrient Components of Culture Media autoclaved with Glucose
C. E. Lankford and Helen Lacy, The University of Texas, Austin and Galveston

Effect of Physical and Chemical Reagents on Rh Antibodies
Sol Haberman, A. F. Reid, and J. M. Hill, Baylor University and Southwestern Medical College, Dallas, Texas

The Precipitation of Poliomyelitis Virus from Human Stools with Methanol
M. Pollard and Joan Connolly, The University of Texas Medical Branch, Galveston, Texas

I. M. Lewis Lecture
Speaker: Dr. Martin' Frobisher, Jr.
Subject: Jungle Yellow Fever

Clinical Evaluation of Darvisul in the Treatment of Acute Poliomyelitis. C. Grulee, G. D. Ford, and F. L. Eldridge, The University of Texas Medical Branch, Galveston, Texas.

The drug N-(2 thiazolyl)-phenyl-sulfonamide (Darvisul), reported by Sanders *et al.*, as being effective in poliomyelitis, was clinically evaluated on human poliomyelitis in The University of Texas Medical Branch Polio Unit in 1948. In a controlled series of 31 patients in the acute stage, there was no correlation between the treated and untreated groups with respect to any shortening of the second stage, to decrease in extent of paralysis, or to any spinal fluid changes. Toxic manifestations were mild, occurring in only two cases, both receiving highest dosages of the drug used.

A similar clinical study last year in North Carolina gave comparable results. Referral was made to a subsequent laboratory study which had been unsuccessful in attempting to verify Sanders' original work.

In conclusion we were unable to demonstrate any beneficial effevt from the use of the Darvisul on acute human poliomyelitis.

Identification of *Coxiella burnetii* in Raw Milk. Joyce Wendt, Audry Buxton, Texas Department of Health, Austin, Texas, and A. B. Rich, City Health Department, San Antonio, Texas.

Coxiella burnetii has been found in the raw milk of naturally infected cows in Australia, Greece, and the United States. In March, 1948, the Texas State Health Department began a search for the presence of *Coxiella burnetii*, the causative agent of Q fever, in the raw milk samples received at the laboratory in connection with routine tests according to Standard Methods. The Q fever organism was identified in 3 of the 73 pools of raw milk of naturally infected cows. These findings involved three dairies in Southwest Texas.

Coxiella burnetii was recovered from the raw milk of an experimentally infected cow as early as 24–72 hours and as

late as the 36th day after inoculation. Evidence showed that the Q fever organism was shed only from the inoculated quarter, and that the infection did not spread to the other quarters of the udder. We wailed to recover *Coxiella burnetii* from the early blood of the infected cow whose serum had a complement-fixing titer of 1:320 on the 33d day after inoculation.

Carbohydrate Metabolism of Filamentous Forms of *Escherichia coli*. Anita Mol and Orville Wyss, The University of Texas, Austin.

Cells of *Escherichia coli* growing on the surface of agar plates were subjected to that amount of ultraviolet radiations that would prevent cell division but permit good growth. The large filamentous forms were collected and washed and their carbohydrate metabolism was compared with normal cells. There was no difference in the concentration of glucose dehydrogenase in the two types of cells when measured by methylene blue reduction tests. However, when succinate and acetate were used as substrates the reduction of the methylene blue by the filaments was less rapid. The differences were most pronounced at pH values away from the optimum. Formate dehydrogenase was more active in the filaments. These quantitative differences in enzyme action may be due to actual differences in enzyme concentration in the injured cells.

Laboratory and Clinical Studies of Polymyxin B and E. Major Edwin J. Pulaski,, Major Hinton J. Baker, 1st Lt. Milton L. Rosenberg, and 1st Lt. James F. Connell, Jr., Medical Corps, United States Army, Fort Sam Houston, Texas.*

Polymyxin is a potent antimicrobial drug in vitro against Salmonella, Shigella, Klebsiella, microorganisms of the coli-aerogenes groups, Pseudomonas, Brucella and some staphylococci, but not against Proteus and streptococci. Resistance in vitro of Pseudomonas was not demonstrated after 27 daily transfers in polymyxin broth. Significant blood levels of polymyxin are obtained after intramuscular injection, but urinary excretion is delayed for about twelve hours. Twenty patients

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with severe complicated gram negative urinary tract infections were given polymyxin for one to six days with good responses in ten, doubtful in seven, and negative responses in three. Dramatic results were obtained against acute Pseudomonas pyelonephritis. Polymyxin is effective topically applied in ridding granulating wounds of susceptible bacteria. Orally administered doses of 200 to 400 mg. a day in water rapidly suppress E. coli but not Proteus, cocci and Clostridia. Parenterally administered, polymyxin causes mild but reversible subjective neurologic disturbances regardless of the dosage employed, while doses in excess of 2.5 mg. per kilogram per day cause renal tubular damage.

Chloromycetin and Experimental Toxoplasmosis. Joy Barnes Cross and Harold Joseph, The University of Texas Medical Branch, Galveston.

Because Chloromycetin is still a very new chemotherapeutic agent, its use in experimental toxoplasmosis is reported at this time in spite of the fact that the number of trials has been small. Quite detailed data regarding toxicity was furnished by the Parke Davis Laboratory and our administration in the diet of 0.15%, 0.2% and 0.3%, respectively, was based on their figures. The diet consisted of 10% sugar, the Chloromycetin and finely powdered dog chow and was fed from baxes designed to permit little or no wastage. No evidence of toxicity at any of the dosage levels investigated was seen.

All infections were produced by subcutaneous inoculation of 0.4 ml. of an emulsion of Tyrode's solution and the brain of a mouse on the verge of dying of toxoplasmosis. The three mice treated with 0.15% of Chloromycetin died in 9 days—one of the untreated mice died at 8 days and the other two at 9 days. With 0.2% of Chloromycetin, one mouse died at 8 days, the other two at 9 days and the untreated mice died at 7 days.

Although the increase in the length of survival in the mice treated with Chloromycetin was too slight to be encouraging, these mice failed to show what had always been an invariable and obvious symptom in toxoplasmosis—drooping eyelids and

faulty vision. Because para amino salicylic acid has been somewhat encouraging in the later stages of toxoplasmosis, it was decided to combine the two—Chloromycetin was fed during the early period (5 days) when the eye defects originate and followed by 1.5% PAS.* One of the untreated mice died at 3 days (much too soon to be attributed to the infection) and the other two at 7 days. Two of the mice treated with 0.3% Chloromycetin died at 8 days and one at 9 days, with no signs of ocular damage. One of the mice treated with the combination of PAS and 0.3% Chloromycetin died at 9 days, one at 10 days and one at 13 days with either slight or no ocular difficulty.

Ideally there should have been larger numbers of mice but the number was limited by the rarity of the test material. The one noteworthy result of these trials is the indication of a protective factor against infection of the eyes. During three years of study of this disease in mice, only those treated with Chloromycetin have been exempt from obviously severe ocular damage before death.

Serologic Applications in Dermatophytosis. William B. Sharpe, The University of Texas Medical Branch, Galveston.

Previously reported precipitation test with fungi has been elaborated to meet additional laboratory situations. Different preparations of antigen and antigen fractions were investigated, and a precipitin absorption procedure devised.

Situations involving the dermatophytes are presented and the manner in which they were met. Certain fungus isolants of confusing morphology were placed by these serologic procedures among the dermatophytes, others were placed in a given species group, and others again in one of two closely related species.

An Unusual Property of the Blood Serum in Rheumatoid Arthritis. Robert M. Pike, S. Edward Sulkin, and H. C. Coggeshall, Southwestern Medical College, Dallas.

It has previously been shown (Meyer, 1922; Waaler, 1940; Rose *et al.*, 1948) that the serum of some individuals with

*All the results of this experiment are listed here although the findings were incomplete at the time of the meeting.

rheumatoid arthritis will agglutinate sheep erythrocytes which have been sensitized with a small amount of antisheep hemolysin. The algebraic difference between the titers of such a serum for unsensitized and for sensitized sheep cells is called the differential sheep cell agglutination titer. Differential titers of 16 or greater were found with serum specimens from 26 out of 66 patients with active rheumatoid arthritis. None of 122 sera from patients with various other acute and chronic diseases and from normal persons showed differential titers greater than 8. When samples of serum were absorbed with sheep cells to remove the normal agglutinins for unsensitized cells, it was found that the agglutinating titer of rheumatoid arthritis serum for sensitized sheep cells was not reduced thereby tending to increase the differential titers of rheumatoid arthritis sera. When 28 samples of rheumatoid arthritis serum which originally had differential titers of 8 or less were retested after absorption, 12 had differential titers of 16 or greater. No titer for sensitized sheep cells greater than 1:40 and no differential titer greater than 8 was found after absorption among 47 samples from normal persons and from diseases other than rheumatoid arthritis except in 2 cases of scleroderma.

The factor responsible for the phenomenon could not be removed from rheumatoid arthritis serum by absorption with sensitized sheep cells. No change in the agglutination promoting property of serum was observed during storage for 3 months at —30° C. or at 4° C. In order to obtain consistent results, however, the amount of rabbit antisheep cell serum used to sensitize the cells must be carefully controlled and can be more accurately measured by means of its agglutinating than by means of its hemolytic properties.

An Investigation of a Smallpox Outbreak in the Lower Rio Grande Valley. J. V. Irons, Joyce Wendt, and Thelma Sullivan, Texas State Health Department, Austin.

An outbreak of small pox in the lower Rio Grande Valley in March-April, 1949, was described. There were nine cases with one death. With one exception none of these cases had ever been vaccinated. The smallpox virus was recovered and identified with specimens from six cases. An intensive vaccination

and revaccination program quickly brought the outbreak under control. The State Health Department Laboratory prepared chick membrane vaccine in combating this outbreak.

Laboratory Aspects of Survey of *Pasteurella pestis* in Texas. Margaret Ann Neville, Dorothy Eben, and J. V. Irons, Sc.D., Texas State Health Department, Austin.

The U. S. Public Health Service and the Texas State Department of Health have been making a plague survey in West Texas. Mr. V. I. Miles, Plague Control Officer, U.S.P.H.S., Brownfield, Texas, has been in charge of field activities. State Health Department entomologists have identified the rodent ectoparasites. Following the finding of *Pasteurella pestis* in Cochran County, Texas, in 1946 the U.S.P.H. Services' Plague Laboratory, San Francisco, laboratory activities have centered in the State Health Department, Austin.

Pasteurella pestis has been found in five species of fleas in Dawson, Cochran, Gaines, and Yoakum counties. From October, 1946, to April 8, 1949, a total of 116,015 fleas were inoculated; 1,571 were included in positive pools. The infective pools contained from 5 to 243 fleas. A total of 3,680 animal inoculations were done, comprising 2,784 ectoparasite pools, and 996 tissue pools. Twenty-three pools of fleas and 4 tissues were found positive for *Pasteurella pestis*. Positive fleas or infected tissues were taken from grasshopper mice, pack rats, and prairie dogs.

THE PRECIPITATING ACTION OF ETHANOL AND ACETONE UPON EGG ALBUMIN*

BYRON M. HENDRIX AND JAMES N. WALKER

The purpose of this paper is to present in a somewhat systematic manner some data on the precipitation and denaturation of egg albumin by ethanol and acetone. These effects of acetone and ethanol have been known in a general way for a long time but it is hoped the data presented here will give a better indication of these effects upon albumin than is available in the literature.

Van Slyke and Heller (1) studied the precipitating action of ethanol upon blood proteins with the hope that this precipitant might be suitable for the removal of proteins in the determination of certain non-protein constituents of the blood. They suggested that zinc chloride be used along with ethanol in order to obtain a more complete removal of certain interfering substances, but it is perhaps not indicated that the zinc chloride is required to bring about the precipitation of coagulable protein. In this work no effort was made to determine the extent of denaturation of the proteins because they were concerned only with the removal of proteins and other interfering substances from the blood. Wu (2) compared ethanol coagulation of protein (albumin) with heat coagulation and concluded that they are essentially the same, in that he believed that denaturation is followed by flocculation in both types of coagulation. This conclusion is at variance with common laboratory experience in that ethanol precipitates egg albumin in a form that may be dissolved in water; this is true, especially if the precipitation takes place at relatively low temperatures and the ethanol is not allowed to remain in contact with the precipitated albumin for more than a brief interval of time. Booth (3) studied the action of ethanol upon hemoglobin and found the coagulation of this protein to be

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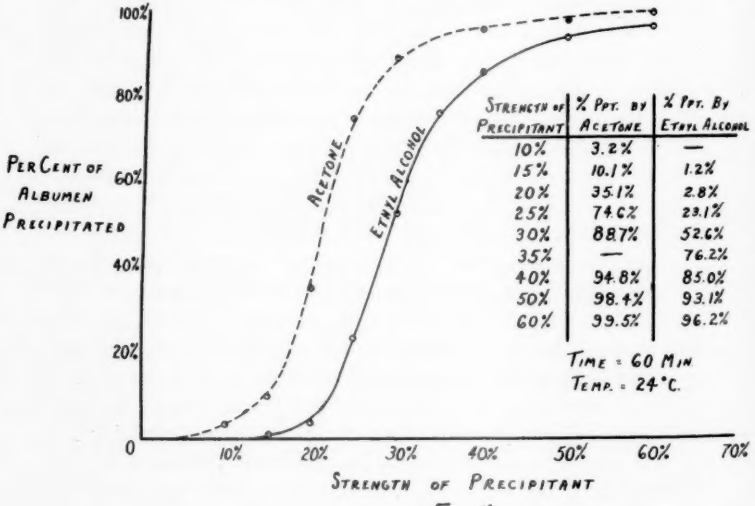
a mono-molecular reaction. The velocity constant of this reaction, according to him, varies with the concentration of the ethanol, the temperature, and in a characteristic manner with the pH. The apparent rate of denaturation decreased on both the acid and the basic side of an optimum pH. Spiegel-Adolf (4) concludes that coagulation and precipitation of proteins by ethanol varies with the type of protein. This of course is to be expected because of the markedly varying solubilities of the various proteins. Teorell (5) finds that the action of ethanol on plasma proteins in the acetate buffers is very characteristic. The ethanol brings about precipitation of plasma proteins at ordinary temperature but on heating, this precipitated plasma protein redissolves in the presence of acetate buffer. He finds this property analogous to the solubility characteristics of Bence-Jones protein in aqueous solution. Egg albumin is in no way affected by acetate buffers. That is to say, ethanol brings about a precipitation of this protein from aqueous solution and this coagulum is not dissolved on heating in the presence of acetate buffers. Ferry, Cohn, and Neuman (6) have shown that 25% ethanol has essentially no denaturing effect upon egg albumin at —5° C. and yet the solubility of egg albumin in ethanol of this concentration and at this temperature is really quite low. The presence of sodium chloride increases the solubility of egg albumin in 25% ethanol at this temperature but under these conditions the solubility is not great. These authors (7) have shown also that 25–35% ethanol does not denature horse carboxy-hemoglobin at —5° C. The solubility of this protein in ethanol solutions of the strength is all but negligible.

It has been known for a long time that acetone brings about the precipitation of many proteins from their aqueous solutions. Weyl (8) (9) showed milk proteins could be precipitated from aqueous solutions by means of acetone and suggested methods of determining the amounts of proteins in milk by using acetone as a protein precipitant. However, the precipitating and denaturing action of acetone upon proteins has not been as extensively studied as the action of ethanol. The denaturing action of acetone upon coagulable proteins has been known for a considerable length of time. Anson and

Mirsky (10) mention it as a well-known denaturant of proteins in their discussion of its coagulating and denaturing effect upon globin.

Methods

Egg albumin crystalized by the method of Kekwick and Cannon (11) was used in this study. The crystals of egg albumin were dissolved in distilled water and dialyzed against distilled water until essentially free from sulfate. All solutions were between 1 and 2% albumin. Careful analyses were made on each solution to determine the exact amount of protein nitrogen present. All precipitations were made close to the iso-electric point of the protein. Acetone or ethanol was poured into the albumin solutions to make the desired per cent by volume of acetone or ethanol. Thorough mixing was brought about immediately by adequate stirring. Aliquot portions of the filtrates were used for determination of the unprecipitated protein and the precipitated protein was calculated by difference. Since the albumin solutions contained such a small amount of electrolyte, it was usually necessary to add a small amount of



Strength of Precipitant	% Ppt. by Acetone	% Ppt. By Ethyl Alcohol
10%	3.2%	—
15%	10.1%	1.2%
20%	35.1%	2.8%
25%	74.0%	23.1%
30%	88.7%	52.6%
35%	—	76.2%
40%	94.8%	85.0%
50%	98.4%	93.1%
60%	99.5%	96.2%

Time = 60 Min
Temp. = 24°C.

Fig. 1.

sodium chloride after the addition of the ethanol in order to prevent the formation of a lyophobic colloidal solution and to bring about prompt precipitation. Attempts were made to filter the solutions of albumin which contained varying amounts of alcohol, without the addition of sodium chloride, but the time required for such filtrations varied to such a considerable extent and colloidal solutions were formed so frequently that constant results could not be obtained. The use of sodium chloride in those solutions containing acetone would not have been necessary but it was used so that the conditions of precipitation of the albumin would be as nearly the same as possible with both reagents.

Results

Figure I shows the precipitation action of various concentrations of acetone and ethanol. Acetone is a more effective precipitation agent for egg albumin than is ethanol. At 25% acetone about 75% of the egg albumin is precipitated after one hour and at 24° C., while at the same concentration time and

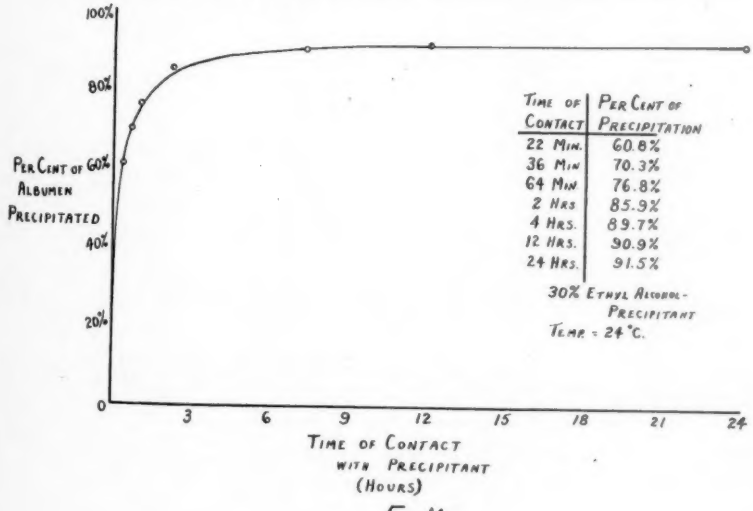


Fig II.

temperature ethanol precipitates only 23%. At the concentration of 60% however, acetone and ethanol precipitate nearly the same amount, namely 99.5% for acetone and 96.2% for the ethanol. Figure II shows however that the precipitating action of ethanol varies with the time. For 30% ethanol the precipitation is less than 70% complete in one hour, but in four hours it is nearly 90% complete and it changes only a little from that amount of precipitation. Enough data are not available to plot a curve for the action of acetone similar to the one in Figure II for ethanol, but what data we have would indicate that 30% acetone brings about the precipitation of nearly 90% of the albumin in one hour and the precipitation is nearly if not quite complete in 24 hours. Figure III shows the effect of varying temperature on the precipitation of egg albumin by ethanol. The data presented here are for a four hour period only and are comparatively low but these results do show that the amount of precipitate increases with the temperature, even when a comparatively low concentration of ethanol is used. Undoubtedly the comparative flatness of the curve results from the low concentration of the ethanol. In

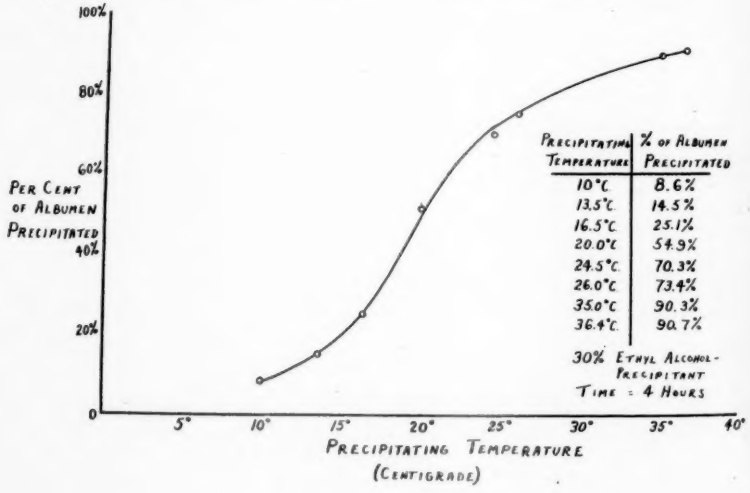


Fig. III.

this case also we lack data to construct a curve for the effect of temperature upon acectone precipitation. Our data indicate that about 90% of the albumin is precipitated by acetone at 24° C. while at the same temperature and concentration only about 70% is precipitated by ethanol.

Figure IV shows that the precipitated egg albumin may be of two types, that which is coagulated and that which has been precipitated without denaturation. In carrying out this series of experiments the albumin precipitated by ethanol or acetone was filtered free from the precipitating medium, suspended in a considerable excess of distilled water, mixed thoroughly and filtered, and the amount of albumin was determined in an aliquot portion of this second filtrate. This albumin was, of course, the reversibly precipitated portion. It will be noted that the amount of coagulated or irreversibly precipitated albumin increases with the temperature and is greater in amount in those samples which were precipitated by acetone. It was shown by Ferry, Cohn, and Neuman that ethanol is not a denaturing agent at —5° C. so our results show

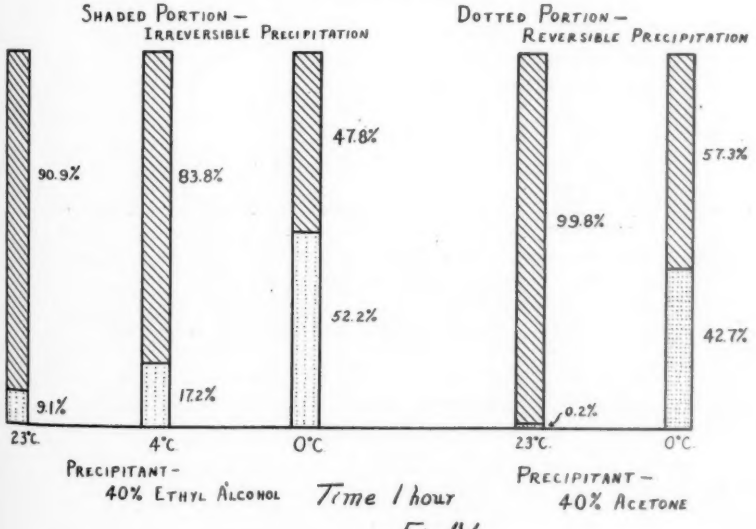


Fig. IV.

a rather marked effect of temperature upon the process of denaturation as well as upon the precipitation.

Conclusions

The results presented above show that precipitation of egg albumin by solutions of ethanol and of acetone varies with a number of factors. Concentration of the precipitating agent, time, and temperature are of importance in affecting the extent of the precipitation.

Acetone appears to be a more effective precipitating agent than ethanol in that acetone brings about a greater extent of precipitation of egg albumin at comparable concentrations, temperature and time.

The reversible precipitation takes place rather extensively at low temperatures, being about half of the total precipitation at 0° C. for 40% ethanol, and somewhat less for 40% acetone, when the protein remained in contact with the precipitating solution for one hour.

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THE EFFECT OF SUBSTANCES KNOWN TO INFLUENCE THE ACTIVITY OF THE NERVOUS SYSTEM ON FIBER OUTGROWTH FROM LIVING EMBRYONIC CHICK SPINAL CORDS*

J. T. Painter, C. M. Pomerat, and D. Ezell

The use of tissue culture technique for micro-pharmacological assays appears valuable because of the ease with which such tests can be performed, the low cost of materials, the rapidity of obtaining results and the opportunity of using genetically homogenous materials. In its simplest form the method utilizes hanging-drop preparations as primary explants. In our experience two technicians can easily set up 300 such cultures in an afternoon. Using suitable end-points results may be read in 24 to 48 hours. Since it is often possible to conduct an entire experiment on explants derived from one animal, the genetic uniformity of the test is enhanced. Methods being perfected by Earle and his co-workers (1948) for the development of a clone from a single isolated cell will extend the opportunity for experimental studies on genetically homogenous material. The perfection of mass culture techniques (Evans and Earle, 1947) opens wide opportunities for biochemical studies on large quantities of cells grown under highly controlled conditions.

Usually drug assay tests utilize explants of embryonic tissues. However, an enormous spectrum of cell types successfully cultivated *in vitro* are available for such work. Many varieties of normal and pathological human material can be utilized. Murray and Stout (1947), for example, have demonstrated that adult human sympathetic ganglion cells, obtained at sympathectomy performed for relief of essential hypertension, migrated and, in some instances, underwent division during a period of several months in lying-drop cultures. Cells in ascitic fluid arising from malignant and non-malignant processes provide an enormous potential of human test material.

*From the Tissue Culture Laboratory and the Department of Neuro-Psychiatry, Medical Branch of The University of Texas, Galveston. Aided by a grant from the United States Public Health Service RG 333C. Received for publication May 28, 1949.

Ivers and Pomerat (1947) have described some of the transformations observed in cultures of peritoneal cells and methods for studying the effect of sterol compounds on these elements have been suggested (Ivers, Pomerat, and Neidhardt, 1948).

The technique of inducing cells to grow directly in medium containing a drug makes for a very stringent method of assay. Conversely, one must also consider that *in vivo* tests usually involve one or more of the effective buffer systems available to the organism for warding off deleterious agents. In cellular terms, the usual LD_{50} represents the quantity of a given drug operating after the organism's capacity to bind, precipitate, and otherwise cope with the agent has been overcome. Such measurements of toxicity represent lethal effects against the first, and presumably, the most vulnerable critical organic system encountered. It appears reasonable that a given LD_{50} might have been different if a more sensitive critical tissue had been in the path of the drug. Moreover, it is very probable that some areas have been adequate in meeting the insult without irreversible injury.

Toxicity tests on explants of particular organs, tissue complexes or perhaps even pure cultures of cells, therefore, may make it possible to assign the locus of injury of a particular drug more specifically. The full extension of this outlook may permit the development of a technique for measuring the capacity of drugs to alter membranes of various species of cells. Leake (1948) has recently urged the importance of evaluating drug action at the molecular level. He has called attention to the importance of considering current concepts regarding the nature of the outer surface of cell membranes (c.f. Davson and Danielli, 1943), to the arrangement of reactive groups, to "openings" between superficial protein molecules which allow for the intracellular entrance of ions, and to the delicate balance of architectonic forces on the membrane as a whole. Drug action may be looked upon as altering surface tension by disrupting surface proteins and dissolving fats in the lipid layer; blockading reactive surface groups resulting in an interference with metabolites; or by altering optimal conditions for enzymatic activity. Growing knowledge regarding the molecular configuration of chemical compounds in

relation to their physiological activity (*biochemorphology*—Leake, 1948) with special reference to hydrocarbon, polar and ionic groups with spacing and stero-chemical factors determining the resulting forces of association, offers an important challenge to the cell physiologist.

It was with the hope of establishing correlations between toxicity *in vivo* and *in vitro* that a program of studies was undertaken in this laboratory on a group of CNS stimulants and depressants. In a comparison of seconal sodium, pentobarbital sodium, sodium amytal, and 14 new barbiturate preparations, good general correlations were found between the convulsive, lethal and anesthetic doses and the minimum quantity required for the inhibition of fiber outgrowth from standard preparations of chick embryonic spinal cords (Pomerat, Drager, and Painter, 1946). However, some marked exceptions were noted. Thus, while the LD_{50} of 3,3-dimethyl allyl ethyl barbituric acid was only 8.84 ± 0.32 mg./kg. in rats, nerve outgrowth, and migration of cells from heart explants was not completely inhibited *in vitro* in the presence of a 1:800 dilution of the drug. In contrast, 3,3-dimethyl allyl allyl thiobarbituric acid which had an LD_{50} of 94.08 ± 8.47 proved the most toxic member of the series in relation to outgrowth from embryonic explants; fibers from spinal cord were totally inhibited at 1:12,800 and all outwandering cells from heart fragments were suppressed at 1:6400.

The present study was undertaken to determine the relative toxicity of clinically useful drugs, known to influence the activity of nerve tissue, with the use of a standardized tissue culture method (Pomerat, Drager, and Painter, 1946).

Materials and Methods

Spinal cords were removed from chick embryos which had been incubated for nine days, using the technique described by Pomerat, Drager, and Painter (1946).

Drugs were selected to represent various well-known schemes of classification including stimulants *versus* depressants, agents affecting the central nervous system or the peripheral nervous system (as parasympathicomimetics, anticholinesterases, sympathicominetics, and autonomic blocking agents). In addition,

various factors which might influence the nutritional properties of the medium such as biotin, folic acid, glutamic acid, and human umbilical cord serum were tested.

These substances were dissolved in Tyrode's solution at strong concentrations and were generally sterilized by passage through a micro-Seitz filter, but in some cases, sterilization by autoclaving for 20 minutes at ten pounds pressure was used. From such stock solutions, serial dilutions were prepared with Tyrode's solution and mixed in equal proportions with embryonic extract derived from chick embryos incubated for seven days. Hanging-drop cultures were made by placing one drop a cockrel plasma delivered from a 27-guage needle fixed to a tuberculin syringe upon a sterile cover glass. A cord explant was placed near the plasma and one drop of the drug-embryonic extract solution was added. These materials were stirred rapidly in order to insure uniform distribution of the components, and the fragment allowed to be fixed in the center of the clot.

In the initial screening process, four such preparations were generally made for each of five dilutions in 1:10 dilution steps. After incubation at 37° C. for 24 hours, the cultures were observed for the length and density of the fiber outgrowth, and for the presence of migrating cells. Similar observations were repeated at 48 hours, when all cultures were fixed in Bouin's solution for staining with the Bodian technique. On the basis of the initial results, experiments were repeated in order to determine the minimum dosage required to produce total inhibition of all fibers and the minimum dosage which produced minimal detectable injurious effects.

A characteristic series of dilutions, illustrating the order of the end-point attempted in the study of cocaine is represented in Table I. In this, it will be seen that the amount of outgrowth was rated and that repetition of titrations was needed for achieving relatively critical values for total and minimal inhibitory effects. Variation was encountered with drugs such as atropine known to be unstable or under conditions where there had been inadequate stirring of the component materials. Most drugs in the series gave very consistent results.

TABLE I

Summary of Experiments on the Effect of Cocaine on the Outgrowth of Fibers from the Spinal Cord of 9-Day Chick Embryos.

Concentration gamma/ml.	Date of Experiment 7–3	7–9	7–17	7–24	8–13	10–2	10–9	10–16	11–4	11–10
12		+++								
16		+++								
20										
25		+++	++++	++++						
28			++++	++++						
33		+	+++	+++						
40										
50		+	+++	+++	+++					
67					++					
80	+									
100					+	+	++			
133						+				
156									++	
200						+				
250							+		+	+
312							+		+	
400	TI*					TI		TI		TI
416							TI	TI		
625							TI	TI	TI	TI
1250								TI		TI
2000										
Control	+++	+++	++++	++++	+++	+++	++++	+++	++++	++++

*TI—total inhibition of nerve outgrowth.

Results

More than 8,000 individual hanging-drop cultures were used to determine the toxicity of 23 different substances known to influence the metabolism of nervous tissue. Table II represents the materials examined together with their physiological action, molecular weight, toxicity *in vivo* for experimental animals and man in comparison with the minimal dose (MID) for the complete inhibition of fiber outgrowth from explants of 9-day chick cord cultures after incubation for 48 hours at 37.5° C.

On the basis of the range of injurious effects *in vitro* from the lowest concentration showing some injury to the outgrowth (lowest injurious dose—L.I.D.) to the minimal dose for total inhibition (M.I.D.), the substances tested fell into six fairly

well defined groups. These groups are listed in Table III in order of descending toxicity. Strychnine, atropine, morphine and lobeline were toxic within the range of 4–100 gamma per ml. of culture media (Group A). In contrast to this group whose toxicity extended over only a short range of concentrations, substances in Group B characteristically showed injury over a considerably wider range (7.5–1250 gamma per ml.). Glutamic acid, chloral hydrate, cocaine, benzedrine, epinephrine, camphor, tetrazol, nicotine, and ephedrine fell into this category. Paraldehyde, coramine and caffeine were similar in showing moderate toxicity—within a range of approximately 250–6000 gamma per ml. (Group C). Drugs showing only slight toxicity (metrazol, sodium bromide, nicotinamide) are listed in Group D. These exerted a toxic effect between 3 and 25 mg. per ml. It will be noted in these groups that with decreasing toxicity, the range of concentrations over which drugs exert deleterious effects shows a greater spread. In Group E, drugs were included in which only partial inhibition was achieved with the highest quantities which could be feasibly incorporated into the medium. This was true for picrotoxin and biotin. Group F contains the remaining substances generally classified as nutrients (para-aminobenzoic acid and folic acid), which showed no injury even in the presence of undissolved crystals.

1. *Strychnine:* This powerful alkaloid generally classed as a stimulant of the spinal cord, has been shown to be excreted 5 minutes after absorption in man. It is known to be more toxic to children although Poe, Suchy, and Witt (1936) found it less toxic to old than young white rats. Continued administration has not been shown to produce tolerance but to establish a more rapid, vigorous response. Equal tetanic doses in frogs produces spasms earlier and of longer duration (Mostrom and McGuigan, 1912).

Sanguinetti (1913) found that strychnine as well as chloral, caffeine, morphine, nicotine, and adrenalin increased the growth of connective tissue associated with cultures of guinea pig peripheral nerve when these substances were placed in tubes adjacent to the explant. Possible stimulating effects were not studied within the scope of our experience. Arkin (1912)

showed that phagocytosis was inhibited by strychnine. This phenomenon is being investigated with tissue culture techniques as described in a paper by Pomerat, Jacobson, and Orr (1949).

A comparison of the toxicity using our tissue culture method with results obtained *in vivo* is difficult in that embryonic material was used, that there is no opportunity for the excretion of wastes in hanging-drop cultures, and finally because there are wide species differences in the susceptibility to strychnine. According to Sollmann, chickens are more resistant to this drug than man. The range *in vitro* is from 6 gamma (1:180,000) to 16 gamma (1:62,500) per ml. of medium and the lethal dose for man is 100 mg. per 75 kg. (1:750,000). While further investigation is necessary, these data suggest that *in vivo* and *in vitro* measurements of toxicity for strychnine may be well correlated.

2. *Caffeine Alkaloid:* While birds are somewhat less sensitive to caffeine than rabbits, or only approximately 150 mg. per kg. (1:15,000), the MID for caffeine alkaloid was 5 mg. per ml. (1:200). Injury was observed, however, at dilutions as low as 1:2500.

It has been reported that the irritability of the CNS in relation to caffeine progresses from above downward. Fragments from the cervical and thoracic regions were not tested systematically. Further work might reveal differences in cultures from various levels of the brain and cord.

3. *Atropine:* This medullary stimulant leads to paralysis at a high dosage. Its toxicity for the child is 10 times that reported for adults. There appears to be considerable variation in tolerance in experimental animals. This appears to be correlated with the protective action of some, as yet unknown, factor in serum and some organs, notably the liver. Kako (1926) noted that the destruction of atropine decreased with thyroidectomy in rabbits. Ikoness and Kusnetzowa (1927) also found this for dogs and claimed that the activity was reestablished by reimplantation of thyroid. Moreover, sera of hyperthyroid patients destroys atropine more actively than normal serum and the reverse for hypothyroids.

Detoxification in the rabbit is believed to be due to rapid destruction of the levo-isomer (demonstrated *in vivo* by von Oettingen and Marshall, 1934). Rabbit serum also binds other alkaloids, especially pilocarpine so that they are undialyzable. Homatropine and scopolamine are also destroyed by serum. Hydrolysis of atropine in various tissues was studied by Bernheim and Bernheim (1938). The esterase is similar to that which attacks ethyl mandelate and is inhibited by physostigmine. It is present in the liver of guinea pigs and frogs and the blood of certain rabbits. Tolerance is increased in infants but is not due to levo-atropine destruction by serum. This is partially explained by increased excretion of atropine in urine (Pilcher, 1934).

Like morphine, habituation is easily achieved. Cloetta (1911) found that non-habituated rabbits completely excrete atropine in two to three days while after habituation, excretion is completed in one day.

Tanneberg (1931) was the first to demonstrate the toxic effect of atropine for the fibroblasts of chick embryos, the MLD being 1:4000. Verne (1948) pointed out that atropine has special interest since the limit of the tolerated dose by the hen is 0.75 gm. per kg., for the rat 0.70 gm. per kg., while for man the value is 0.002 gm. per kg. Using atropine sulphate Verne showed that the toxicity *in vitro* was the same for cells from hens, rats, and humans. All cellular activity was inhibited at 1:1500 while at 1:3000, only slight growth was observed with relatively rare mitoses, the cytoplasm being filled with lipoid granulations. Cultures containing 1:5000 grew like the controls. Renal tissue and nervous tissue did not present special sensitivity (Verne, 1948).

In our own experience, tests using atropine sulfate gave an unexplained high variability of results. Approximately 400 culture tests performed in 8 different series yielded average values indicating much higher toxicity than that reported by various authors, the LID being approximately 4 gamma and the MID 40 gamma per ml. Cockrel plasma used in our experiments was not always derived from starved animals. This factor, as well as possible variations arising from the use of plasma of different individuals is under investigation. Using

human plasma in relation to cells from different animals, Verne (1948) found that the toxicity remained constant.

According to Spadafina (1933), atropine sulfate, unlike strychnine, tends to induce macrophagic transformations from fibroblastic elements. Chevremont (1949) has reviewed the problem of macrophage induction *in vitro* by various drugs. This question is also under investigation in our laboratory.

Verne (1948) found that fibroblasts could be adapted to atropine after 48 hours in a culture containing 1 pp. 5,000 to extent that they could tolerate 1 pp. 3,000 when subcultured regardless of plasma utilized. Moreover, he found that this was true for other strains of cells. The question of drug adaptation will be discussed in the section on morphine.

4. *Picrotoxin:* According to McNally (1937), the fatal dose from pure picrotoxin has not been reported but death occurred 30 minutes following the injection of 36 grains of the powdered berries of *Anamirta paniculata*, which would be equal to about one-third of a grain of picrotoxin. Sollman reports this amount to be toxic but does not state that it is the fatal dose. According to Kohn-Abrest (1934), the fatal dose is 125 mg. Assuming that this is correct, observations in tissue cultures revealed the surprising fact that at the highest concentration which could be dissolved without marked crystal formation in the medium (3,750 gamma per ml.) not more than 50% inhibition of outgrowth was observed. Even when crystals were incorporated in the plasma clot, fibers were seen to migrate to a degree rated as one plus.

These observations are curious in light of the fact that picrotoxin is known to act as one of the most powerful convulsants of the medullary area. While stimulation is more intense in the higher centers, spinal stimulation is also involved. Thus while the cord is known to be susceptible, fiber outgrowth in embryonic chick explants appeared remarkably resistant. Cultures containing approximately 500 gamma per ml. of picrotoxin resembled controls. One must assume, therefore, that the toxic effects exerted by picrotoxin are due to interference with the function of the fiber rather than due to the destruction of the cells. The combined use of chloral, morphine, and

minimal doses of atropine as an antidote strengthens this conclusion in that the therapeutic objective appears to be the counteraction of the asphyxia induced by the powerful convulsions produced with picrotoxin. Conversely, picrotoxin has found an important place in the treatment of desperate cases of barbiturate poisoning by as much as 39 grams of phenobarbital. Further investigations with *in vitro* techniques might be directed toward the study of picrotoxin injury to mitochondria since these bodies have been reported to act as bearers of respiratory enzymes.

5. *Lobeline:* Intramuscular injection of from 0.5 to 5.0 mg. per kg. results in a marked increase in respiration and slowing of the heart accompanied by a rise in blood pressure. The effects of this drug are analagous to those produced by nicotine and are caused by stimulation of the carotid sinus. According to data deposited with the National Research Council, the LD_{50} for the rabbit is 10 mg. per kg. given intravenously (1:100.000). Lobeline is classed in Table II as a member of Group A in that it showed a high toxicity and injury within a relatively narrow range of concentrations. Some injury was observed at somewhat more than 50 gamma (1:20,000) while total inhibition was obtained with approximately 100 gamma (1:10,000). Although existing data for the toxicity of lobeline *in vivo* is only obtainable for the rabbit, our *in vitro* observations refer to embryonic chick tissue. The fact that toxicity *in vivo* appears ten times greater than in the tissue culture makes it very probable that poisoning effects from this drug may operate via some other vulnerable tissue or as a secondary result from its action on the respiratory center.

6. *Coramine:* This drug like metrazol is a medullary stimulant but having, in addition, the capacity to excite the midbrain and the spinal cord (Kohlhoff, 1928). It is similar to picrotoxin and camphor in producing irregular convulsions. Coramine stimulates the carotid body center and is used clinically as a respiratory stimulant at a dose usually close to that producing convulsions (Cramp, 1927; Massart, 1930).

In vitro it showed injury over a wide range of concentrations, the quantity required for total inhibition being 6.25 mg. per ml. while the L.I.D. was 2.1 mg. per ml.

TABLE II

Summary of Toxicity of Substances Tested with Tissue Culture Technique.

Drug	Classification	Mol. Wt.	Minimal Inhibitory Dose for Spinal Cord Culture (Mid)	Toxicity *In Vivo*
1. Strychnine	Stimulant of spinal cord	334.40	16 gamma	[8]Man: 100 mg.; recovery after 250 mg. with treatment. [8]Cats, Dogs: 0.75 mg. per kg. [8]Chicken: 30–40 mg. per kg. P.O.
2. Caffeine Alkaloid	Stimulant of cord and psychic centers	194.19	5,000 gamma	[8]Man: over 10 gm. [8]Rabbit: over 150 mg. per kg. [8]Pidgeon: under 150 mg. per kg. [6]Rat. LD_{50} 200 mg. per kg. P.O.
3. Atropine	Stimulant of medulla, midbrain, motor cortex	289.36	40 gamma	[8]Man: 0.1 gm. for adults; 0.01 gm. for child. [6]Rat: LD_{50} 2,500 mg. per kg. subcut.
4. Picrotoxin	Stimulant of medulla and midbrain	602.37	over 3750 gamma with 1 plus nerve outgrowth	[3, 6]Man: 125 mg. fatal; 20 mg. toxic. [8]Rabbit: LD_{50} 1.2 mg. per kg.; LD_{85} 1.5 mg. per kg. I.V. [8]Dog: 1.5 mg. per kg. I.M. fatal; 0.3 mg. per kg. I.V. convulsive.
5. Lobeline	Stimulant of medulla	321.40	98 gamma	[6]Rabbit: LD_{50} 10 mg. per kg. I. V.
6. Coramine	Stimulant of medulla	152.22	6,250 gamma	Not known
7. Metrazol	Stimulant of medulla	138.17	12,500 gamma	Man: 500 mg. fatal[2]; 0.1–0.3 gm. convulsive.[8] [1]G. Pig: LD_{85} 75 mg. per kg. I. P. [6]Rat: LD_{50} 75 mg. per kg. subcut.
8. Benzedrine	Stimulant of medulla, sympathicomimetic	135.20	500 gamma	[8]Man: 125–150 mg. daily for 4 years without effect. [6, 8]Rats: MLD 25–35 mg. per kg. subcut.

9	**Ephedrine**	Stimulant of medulla, psychic centers; sympathicomimetic	183.25	830 gamma	[8]Man: 400 mg. nontoxic. [6]Rat: LD_{50} 135–140 mg. per kg. I.V.
10.	Epinephrine	Sympathicomimetic	183.20	250 gamma	[8]Man: 10 mg. subcut. fatal. [8]Rabbits, Dogs, Cats: 0.1–0.6 mg. per kg. I.V.; 2.5–10 mg. subcut.
11.	Nicotine	Stimulant of medulla	162.23	830 gamma	[8]Man: 60 mg. pure nicotine, 2 gm. tobacco fatal. [5]Dog: LD_{50} 10 mg. P.O.; LD_{100} 3 mg. I. V. [5]Cat: 1.3 mg. per kg.
12.	Camphor Tetrazol	Stimulant of medulla and psychic centers	192.12	1,250 gamma	[8]Man: 0.7–1.0 gm. fatal in children; 2 gm. toxic for adults. [6]G. Pig: LD_{50} 150–180 mg. per kg. P.O.
13.	Cocaine	Stimulant of motor cortex and psychic centers; sympathicomimetic; local anesthetic	303.35	400 gamma	[8]Man: 1.2 gm. fatal but marked variability. [8]Cats: MLD 15 mg. I.V. [1]Mice: MLD 85 mg. per kg. I. P. [6]Rats: LD_{50} 100 mg. per kg. I. P.
14.	Morphine	Cerebral depressant; stimulant of spinal cord and medulla	303.18	80 gamma	[8]Man: 200–400 mg. fatal; death reported from 60 mg. [8]Rabbits: 100 times fatal dose for man. [8]Pidgeon: 10 times fatal dose for man. [6]Rat: LD_{50} 42 mg. per kg. cubcut.
15.	Sodium Amytal	Depressant	226.27	31,000 gamma	[8]Man: MLD 1.6 gm. [6]Rats: LD_{50} 230 mg. per kg. subcut.; 140 mg. per kg. I.M.[8]
16.	Seconal Sodium	Depressant	222.12	15,500 gamma	
17.	Pentobarbital	Depressant	226.10	31,000 gamma	[6]Rat: LD_{50} 120 mg. per kg. subcut.
18.	Sodium Bromide	Depressant	102.90	20,000 gamma	[8]Man: 300 mg. dangerous; 200 mg. with psychosis; [1]recovery with serum bromide of 350 mg. %. [8]Rabbits: 2.3–3.2 gm. per kg. I. V. with deep narcosis. [6]Rats: LD_{50} 3,500 mg. per kg. P.O.

19.	Chloral Hydrate	Depressant	165.42	750 gamma	[5]Man: 10 gm. fatal (variable); 1–2 gm. with hypnosis. [6]G. Pig: LD_{50} 500–500 mg. per kg. I. V.
20.	Paraldehyde	Depressant	132.16	2,500 gamma	[5]Man: 100 gm. toxic; 150 gm. survived. [1]Rabbits: LD_{50} 0.45 ml. per kg. [1]G. Pig: MLD 12.5 ml. per kg. of 1:10. I.P. [1]Cats: LD_{50} 0.45 ml. per kg. [1]Rats: MLD 12.5–13.5 ml. per kg.
21.	Insulin, Protamine Zinc Insulin	Medullary hormone of adrenal gland	35,000	2 plus outgrowth of nerve fibers with 10 units	[4]Man: 1,800 units for 60 kg. man; coma at blood sugar level of 0.035%.[8] [1]Rats: 20–40 units per day without effect.
22.	Nicotinamide	Vitamin B complex	95.04	25,000 gamma	None.
23.	Biotin	Vitamin B complex	244.30	inhibition of outgrowth in presence of crystals	None.
24.	Folic Acid	Vitamin B complex	398.24	None	[7]Man: 150 mg. I.V. with shock-like state. [7]Rabbits: LD_{50} 410 mg. per kg. [7]Rats: LD_{50} 500 mg. per kg. [7]G. Pig: LD_{50} 120 mg. per kg. [7]Mice: LD_{50} 600 mg. per kg.
25.	Glutamic Acid	Vitamin B complex	147.13	750 gamma	None.
26.	Para-aminobenzoic Acid	Vitamin B complex	137.13	None	None.

[1]Andersen, *et al.*
[2]Hassin.
[3]Kohn-Abrest.
[4]Liebert and Weil.
[5]McNally.
[6]National Research Council.
[7]Sargent.
[8]Sollmann.

7. *Metrazol:* In his excellent discussion of this drug's action on the C.N.S., Hildebrandt (1937) cites the work of Schoen (1926) who found that removal of the cerebrum above the thalamus did not affect convulsions. This result was confirmed by Winniwarter (1937) who used the pidgeon. Schoen (1926) also noted that the convulsant dose was three times increased in the decerebrate rabbit. In the same year, Blume (1926) concluded that in cats, convulsions were due to the afferent impulses in the reflex arc. He also found that the spinal cord was only slightly less sensitive than the entire central nervous system. Koll (1937) reported that in normal animals, the site of action for metrazol lies below (ventral) to the pyramidal tract in the motor apparatus and that abolition of narcosis is due to a true antagonism between analeptics and narcotics.

At the cellular level, Hassin (1939) concluded that in the human brain, especially in the olivary body, barbiturates, insulin, and metrazol produce morphological changes in cerebral ganglionic cells often associated with neuronophagia but without marked microglial or macroglial reactive phenomena. These changes are not interpreted as being specific for these drugs, but resemble the action of a variety of inorganic and organic poisons. In a more searching experimental analysis, Liebert and Weil (1939) found that the brain of rabbits receiving less than 750 mg. of metrazol showed ". . . mild vacuolation of the cytoplasm of the different neurons together with mild hypertrophy and hyperplasia of all these types of glial elements. With increasing doses, length of treatment and time of survival, the picture changed to shrinkage of both cytoplasm and nuclei, with folding of their membranes, and there was a diminution in the number of neurons, with increasing degrees of neuronophagia. The hypertrophy and hyperplasia of both astrocytes and microglia cells, too paralleled the dose injected and the time of survival." These authors call attention to the resemblance of their histopathological findings with observations made on human brains in cases of epilepsy and of vascular disease. They conclude that the mechanism of injury by metrazol involves an initial sudden contraction of cerebral

arteries followed by hyperemia, but caution against the conclusion that the histopathology can be explained entirely upon the basis of the anemia consequent to these two mechanisms.

With the use of his precise, direct method of observing cellular behavior in the transparent tissues of the tadpole's tail, Speidel (1940) observed disruption of synapses following treatment with metrazol. The restoration of synaptic connections following reversible injury effects, led this author to suggest that the therapeutic results achieved with the use of this drug in psychiatry might be attributed to the opportunity for the establishment of new synaptic pathways. .

Like other powerful stimulants useful in antagonizing the effect of barbiturates, such as picrotoxin and coramine, metrazol was also found to have a surprisingly low toxicity when measured with our tissue culture technique. Some injury was observed in the presence of 7.5 mg. per ml. of culture medium while 12.5 mg. per ml. or 1:80 were required to achieve total inhibition of outgrowth. Taking 500 mg. (Hassin, 1939) as the fatal dose, one should expect that, on the basis of a 75 kg. man, the relative dose would be 1:150,000 or approximately 1,900 times more toxic than that found for cells *in vitro*. Obviously, in spite of the differences in modes of measurement, the sensitivity of the living organism to metrazol depends upon reactions peculiar to the organism as a whole. Under such conditions, the interplay involving equilibria in a highly complicated system of respiratory ferments and other precisely balanced physico-chemical systems accounts for the great vulnerability to metrazol. The situation obtained *in vitro* involves a considerable simpler order of physiological processes.

Further studies *in vitro* should be carried out with the hope of obtaining evidence of cytological changes as a result of the antagonistic action of metrazol to such substances as barbiturates, paraldehyde, chloral hydrate, urethane and novocaine.

8. *Benzadrine:* The effect of this drug in man is said to be the facilitation of the flow of thought at the expense of concentration and has been reported to improve visual acuity for as long as six hours after administration (Lebensohn, 1944). The physico-chemical basis for this action, according to Mann and Quastel (1940), is the neutralization of the depressant

effect of tyramine and other amines on the respiration of brain tissue as found in studies on brain slices in dextrose solution.

The MLD for the rat has been reported to be between 25–35 mg./kg. when given subcutaneously. In contrast, this drug appears remarkably well tolerated in man, 125–150 mg. daily for a period of four years having produced no physical ill-effects according to Shorvon (1945). *In vitro* studies utilizing tissue culture techniques revealed some injury to fiber outgrowth at 80 gamma per ml. with total inhibition achieved at 400 gamma per ml. These tests showed benzedrine to fall within a range similar to that obtained for other sympathicomimetics—the LID for ephedrine, closely related in chemical structure, being near that of benzedrine but the quantity needed for total inhibition approximately 40% greater. Cocaine was also toxic over a similar dilution range.

In view of the relatively high toxic properties exhibited *in vitro*, even though based on tests utilizing embryonic chick tissues, it would seem that the relative tolerance of this drug by man must depend upon an effective detoxifying mechanism. Excretion studies seem to bear this out in that 9% of the drug is eliminated in 24 hours, 50% in 48 hours, the remainder apparently being inactivated by the body.

9. *Ephedrine:* Since ephedrine resembles epinephrine in its chemical structure and physiological effects with the exception that it is not sensitized by cocaine and denervation (Tainter and Chang, 1927), it is interesting to find that its M.I.D. (830 gamma per ml.) is more than three times that of epinephrine in our *in vitro* tests. Verne (1948) found that 200 microgamma per ml. produced important changes in the cytoplasm characterized by vacuolization but without showing an influence on mitosis. Seevers (1930) found that large doses, administered over long periods of time to animals, produced deleterious and maniacal phenomena resembling cocaine effects, especially when the animals had been habituated to this alkaloid. It would be interesting to study the cytoplasm of nerve cells following such injuries.

10. *Epinephrine:* This naturally occurring sympathicomimetic hormone was included in this study for comparison with

benzedrine, ephedrine, and cocaine. The fatal dose for man is given by Sollmann (1939) as 10 mg. (1:7,500,000) when administered subcutaneously. Verne (1948) found that at 100 gamma per ml. epinephrine produced effects resembling those of colchicine and that this effect depends upon its oxidation. If it is protected by vitamin C or glutathione, adrenalin has no effect. He states further that adrenalin is inactive in relation to cancerous tissues suggesting that these contain reducing substances, notably vitamin B.

The L.I.D. for epinephrine was 30 gamma and the M.I.D. was 250 gamma per ml. of culture medium (1:4000). It is considerably more toxic than cocaine, benzedrine and ephedrine. The greater toxicity of this drug *in vivo* (1,875 times) reflects the vulnerability of the extensive sympathetic system and its violent effects on the body equilibria.

11. *Nicotine:* With a fatal toxicity of 60 mg. for man, this was the most powerful poison studied in this report. A measure of its effect is suggested in Sollmann's note that ". . . vapor arising from a glass rod moistened with it and brought near the beak of a small bird causes it to drop dead at once." Symptoms of nicotine poisoning resemble those of hydrogen cyanide or those of asphyxia, acting primarily upon the medullary center and producing cardioaortic and sinocarotid reflex disturbances similar to the effect of lobeline.

It is generally believed that nicotine acts in the region of the end plate (Sollmann, 1939), but Ellis, Thienes, and Wiersma (1942), working with crustacean nerve-muscle preparations found that 1% nicotine was without apparent effect. Wiersma and Schallek (1947), have studied the action of various drugs in relation to synaptic block by nicotine on the giant fibers of the crayfish. These authors conclude that a mechanism involving (1) blocking of synapse by nicotine is followed by (2) a protecting effect when certain structurally competing drugs are used. This is believed to demonstrate that a change takes place in the nicotine molecule which stops its action but that it remains attached by a firm bond as a protective mechanism. At the chemical level, Baker, *et al.* (1938) give evidence that tissue oxidation is checked by nicotine

through its effect on lactic and pyruvic acid, less on dextrose and practically none on succinic acid.

While it must be accepted that man is readily habituated to this noxious substance, Behrend and Thienes (1933) found that adult rats did not become tolerant but that young rats developed definite tolerance to minute convulsive doses and to conditioned salivation reflexes. Dixon and Lee (1912) claim that the liver of habituated animals destroys nicotine somewhat more rapidly than that of controls, however, Edmunds (1916) failed to confirm this conclusion. Hatcher (1904) did not find that the serum of habituated animals could protect other animals against intoxication.

Nicotine was intermediate between ephedrine and camphor tetrazol in the pattern of its toxic effect *in vitro*. Some injury was seen at 250 gamma per ml. while 830 gamma produced total inhibition. In the light of its highly poisonous nature *in vivo*, these values obtained in the tissue culture tests do not appear proportionately high.

12. *Camphor tetrazol:* With a fatal dose of 0.7–1.0 gm. for man, camphor has not proven safe for convulsive procedures. Its tetrazol derivative has been shown to be even more dangerous. Unlike other convulsants of medullary centers (e.g., metrazol or picrotoxin), camphor tetrazol proved somewhat more toxic and fell into Group B (Table III) with an L.I.D. of 625 gamma per ml. and an M.I.D. of 1,250 gamma.

13. *Cocaine:* Upon the well-known pattern of facts such as the capacity of cocaine to paralyze all nervous tissues, the greater sensitivity of sensory fibers, and the prompt, complete return to function following the removal of the drug, Lorente de Nó (1947) has pointed out that this compound is a true nerve anesthetic. In contrast to ether which alters membrane potentials, cocaine ". . . changes very little all those properties of nerve that are accessible to analysis by means of electrical measurements." On this basis, he urges that a study of the effect of cocaine on the respiratory exchange of nerve would be of great value.

The range of toxic effects for cocaine being in the same general category as that found for sympathicomimietic amines,

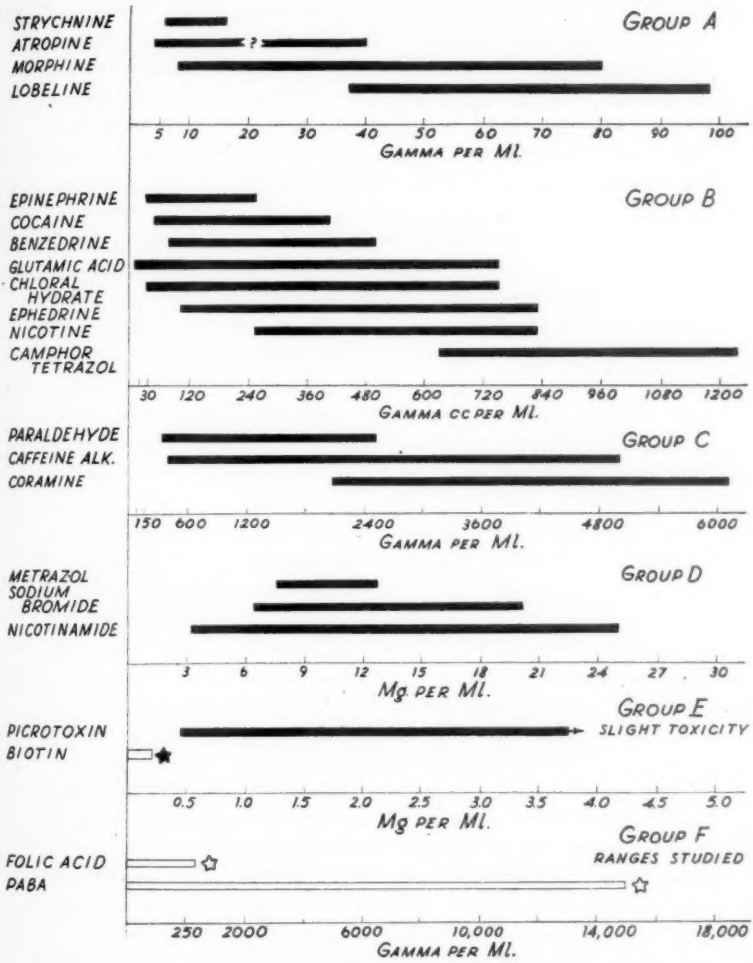

TABLE III

Toxicity of various substances when tested on the outgrowth of fibers from explants of 9-day chick spinal cords after 48 hours of incubation. The minimal injurious dose is indicated by the value at the left side of each bar while that indicated at right refers to the minimal quantity producing total inhibition of outgrowth. Bars not represented in solid black indicate range of concentrations tested but showing no toxic effect. The black star represents inhibition in the presence of crystals. White stars indicate no injury in the presence of undissolved crystals.

the M.I.D. was 400 gamma while the L.I.D. was 50 gamma per ml. of culture medium. The fatal dose for man is given by Sollmann as 1.2 grams but involves individual variation. This value can be expressed as 1:62,500 while that found for the chick cord is 1:25,000. It is likely that in human poisoning, much of the drug is destroyed or inactivated before reaching nervous tissue in sufficient concentration to exert fatal effects.

According to Gurd and Sacks (1939), epinephrine 1:50,000 increases the toxicity of cocaine in white mice. Ephedrine decreases cocaine toxicity at 1:1000 and increases it only above 1:200 (Graham and Gurd, 1940). Dogs habituated to cocaine are hypersensitive to ephedrine and show increased tolerance to morphine sulfate (Downs and Eddy, 1932; Stanton, 1938). Such relationships might well have been explored at the cellular level with tissue culture technique but could not be included within the scope of the present report. (See discussion under Morphine.)

14. *Morphine:* In addition to the fact that the fatal dose for man is from 200–400 mg., although it has been recorded for as little as 60 mg., it is important to note that destruction, primarily in the liver, proceeds slowly in the body—one-third or more of the total dose remaining after 24 hours. However, injection into fertilized eggs with incubation causes no destruction of morphine sulphate or derivatives if the embryo dies before it is half developed. If development is complete, heroin is destroyed completely, morphine sulphate very largely, codeine and dionine none at all. Destruction apparently proceeds only after a certain amount of development has taken place, perhaps of the C.N.S. Habituation does not appear to alter the destruction rate materially (Hatcher and Gold, 1929). The toxicity of morphine sulfate for newborn rats is ten times that for the adult and reaches the adult level at 21 days (Gibbs and Bobb, 1938). This type of phenomenon may be explained by the fact that a milk diet increases the toxicity of morphine for white rats (c.f., Hunt, 1910). Hunt (1905, 1907) also found that feeding with thyroid extract or iodine increases the susceptibility of white mice, rats, and guinea pigs to morphine sulphate. Meltzer (1913) concurs with this view but further

states that the depressive phase of this drug's action is increased whereas the tetanic effect, and therefore the fatality, was decreased with iodine in these animals.

The action of morphine on the spinal cord causes an increase in the spinal reflexes and, in frogs, may give convulsions indistinguishable from those produced by strychnine. In addition, McGuigan and Ross (1915) noted that the injection of morphine into frogs sensitized these animals to strychnine so that tetanus from the latter developed more quickly if one-half to two hours elapsed between the administration of the two drugs. Pulewka (1927) has also stated that the toxic effect of picrotoxin is increased by morphine in white mice.

If one assumes an average MLD of 300 mg. of morphine for man (1:250,000), the corresponding value obtained with our test was 80 gamma per ml. or 1:12,500. Some injury was produced with as little as 8 gamma per ml. or 1:125,000 and, therefore, this drug is included along with strychnine, atropine, and lobeline (Group A) as the most poisonous members of our series when tested directly upon cord explants. It may be reasonable to expect from these findings that the vulnerable mechanism in the nerve cell may be the same for each member of this group of drugs.

In 1938 Sasaki established that fibroblastic cultures could be adapted rapidly to strong doses of morphine. If this drug is suddenly eliminated from the medium used in cultivating such a strain, diminution of growth rate and degeneration was observed. Cultures habituated to high concentration of morphine died if the drug was suddenly excluded from the medium. These results have been confirmed by Kubo (1939) who also showed that dishabituation could be easily demonstrated for codeine, somewhat less easily for heroine and achieved with some difficulty with eucodal.

Habituation has been the topic of several important studies using *in vitro* techniques. Thus, progressive tolerance to copper sulfate; sodium arsenite (Vollmar and Li, 1940); dinitrophenol and atropine (Verne, 1948) has been demonstrated. In view of these studies one is forced to conclude that habituation is essentially a local, cellular phenomenon. This interpretation has been ably discussed by Verne (1948). Much additional

work might be done in examining the reciprocal interrelationships of various drugs altering the sensitivity of nervous tissue to their effects as pointed out in various sections of this paper. For example, it might be possible to demonstrate that an increase or a decrease in the cellular reaction to a given drug might not be affected unless a variety of cell species was present.

15, 16, 17. *Barbiturates:* The fatal toxic dose for commonly used members of this series of compounds is well established for man (generally ranging from 1.5 to 2 gms.—see Table II). While excellent reviews such as that of Tatum (1939), Sollmann (1939) and others have dealt with the fate, destruction, location of action, and habituation, few studies have been concerned with the toxicity of the barbiturates at the cellular level.

The great clinical importance of this large group of depressants has produced an enormous series of compounds which offer an exciting challenge for the study of the relation of chemical configuration to biological activity. An attempt to explore this field (Pomerat, Drager, and Painter, 1946) with the use of tissue culture technique has been discussed in the introduction of this paper. While no systematic effect was observed in relation to the position of any particular radical, a much larger series of compounds should be examined. Higher toxicity to nervous tissue might be associated with molecular configurations related to 3,3-dimethyl allyl allyl thiobarbituric acid. In contrast, the pattern of 3,3-dimethyl allyl ethyl barbituric acid could serve as a starting point for the synthesis of compounds characterized by little injury *in vitro*. Total inhibition of fiber outgrowth was obtained with 15.5 mg. per ml. for seconal, and 31 mg. per ml. for both pentobarbital sodium and sodium amytal. In a previous study Pomerat, Drager, and Painter (1946) found that heart fragments appeared somewhat more resistant to the deleterious effects of barbiturates than were corresponding cultures of embryonic cord, suggesting that in the survey of drug toxicity, representative tissues should be studied in addition to estimates of gross lethal effects.

18. *Sodium Bromide: In vitro* experiments are correlated with the well known relatively low toxicity of this drug, the LID being 6.25 mg. per ml. while 20 mg. per ml. were required to prevent all fiber outgrowth.

The mechanism of action is not known but the bromide content of any organ has been reported by Frey (1937) to be in indirect ratio to the chloride ion in the extracellular fluid and only on long continued administration are cells found to take up bromide ions and this is not related to the intracellular chloride. The mechanism of narcosis is believed to be related to the direct action of bromide ions on the cell surface as it does not readily penetrate the cell membrane. Bromides cause diminution of alpha waves of the electroencephalogram indicating cortical depression (Berger, 1934).

19. *Chloral Hydrate:* While the fatal dose for man is generally set at approximately 10 gm., recovery has taken place from as much as 28 gm.; and death has occurred with as little as 1 to 3 gm. (McNally, 1937). The mechanism of injury appears to result from the paralysis of the respiratory center, although it is suggested that cardiac paralysis of an already weakened heart may cause death.

In vitro, the toxicity of this drug placed it in Group B with a range of injury effect resembling glutamic acid quite closely, the MID being 750 gamma per ml. while the LID was 30.

20. *Paraldehyde:* This useful but unattractive hypnotic with variable toxicity for man was found to inhibit the outgrowth of fibers from explants when 1 pp. 400 (2.5 mg. per ml.) of the USP preparation was contained in the medium. Some injury was noted in the presence of as little as 1:4000 (0.25 mg. per ml.). Paraldehyde is generally considered relatively nontoxic in clinical usage, therefore, the position of this drug in Group C appears somewhat anomalous. It is possible that its highly volatile nature is somehow involved in its toxic manifestations *in vitro*.

21. *Insulin:* The convulsions due to high dosaging of this drug are believed to involve changes in intracranial pressure

since the cysternal pressure rises rapidly in dogs (Sollmann, 1939). Oxygen consumption of the brain is reduced and the immediate cause of death is generally attributed to respiratory failure. In a report on death from insulin shock, Hassin (1939) found pronounced rarefaction or edema of the tissues. Weil, Liebert, and Heilbrunn (1938) determined the toxic dose for the rabbit and computed that this corresponded to a total of approximately 1,800 units for a patient weighing 60 kg.

Liebert and Weil (1939) have postulated that insulin appears to prevent the utilization of oxygen as a result of dextrose deprivation of the neurons. In a comparison of insulin versus metrazol injury, these authors state that ". . . in the first group the cerebral cortex was earlier and more severely affected while in the second group the striatum and hippocampus showed the more marked changes. When equal periods of treatment are compared, it seems that the diminution in the number of neurons, the hyperplasia and hypertrophy of macroglia and microglia and the neuronophagia were much more pronounced in the second group."

As reported in a preliminary note (Painter and Pomerat, 1948) crystalline and protamine zinc insulin at 10 units per ml. permitted outgrowth of fibers resembling corresponding untreated control cultures for the first 24 hours but by 48 hours it was evident that these elements were arrested. Stained preparations revealed only fibers of relatively large diameter, the dense network of fine fibers being almost completely eliminated. It would seem, therefore, that the delaying action of protamine and zinc on insulin is not demonstrable under the condition of the experiments and is probably associated with the complex detoxifying and excretory mechanisms of the whole organism. The arrest of fiber outgrowth after 24 hours in the presence of insulin may have been due to excessive dextrose destruction. Unfortunately microchemical determinations were not made nor was a systematic addition of glucose attempted as a compensatory device to make up for probable carbohydrate depletion.

On the basis that the approximate lethal dose of insulin of 1,800 units for a 60 kg. man (Weil, Liebert, and Heilbrunn,

1938), 33 units would be required to destroy one gram of tissue. While we could not introduce a sufficient number of units into the culture medium to achieve total inhibition, it may be assumed that since 10 units of insulin resulted in 50% inhibition, the toxicity *in vivo* and *in vitro* appear to be roughly of the same order of magnitude.

Vitamins

An analysis of the toxic effect of nutrient factors at high concentrations or, as in the case of amino acids, in improperly balanced combinations invites special efforts in the use of *in vitro* techniques. Little data is available for the fatal dosages of these substances. Since the time of the notable work of Baker (c.f. Parker, 1938), the preparation of a synthetic medium for the animal cell has constituted a problem of basic importance. The achievement of White (1943) in the development of a nutrient formula for the plant cell has intensified efforts in this direction. Workers in the field of cellular biology look to the possibility of exploring the special requirements of nervous and other cell types in health and disease when a basic synthetic medium is available. No attempt was made within the confines of this study to do more than examine certain vitamins in the B complex which had proven of interest in related studies.

One of the complicating factors in the analysis of the action of vitamins in tissue culture tests is the fact that embryonic extract ordinarily used in the formation of plasma clots is rich in vitamins and, therefore, if it is used, it must be remembered that it has contributed to the total quantum. In an important contribution in this field, Hetherington (1946) avoided the use of embryonic extract. This author notes that the literature relating specifically to the action of vitamins on cells *in vitro* is practically non-existant. Hetherington (1946) reported that concentrations of more than 1,000 gamma per ml. of medium of the individual B vitamins were detrimental to growth of skin and brain and, with the exception of thiamine, to the heart. Another general conclusion was that the B vitamins prevented degeneration as compared with untreated controls—"This was most noticeable in brain cultures with

biotin and folic acid in which the controls were badly degenerated after five days whereas the vitamin cultures, especially those containing folic acid, survived an additional 7 days." Hetherington's procedure consisted of finding the minimum quantity of the various vitamins required to achieve "equivalent or better growth" of brain, heart, and skin than corresponding cotnrols.

22. *Nicotinamide* (Niacinamide, Nikethamide): There is little evidence that this compound has any marked specificity for nerve cells. It is known to be without influence upon the polyneuritis typical of pellagrous patients. In spite of its role as an essential factor in the diet, it was found to show some damage to nerve fibers with as little as 3 mg. per ml. while 25 mg. per ml. proved totally inhibitory to outgrowth of cord fragments. In its position in Group D (Table III) it is seen to have a range of toxicity only slightly greater than that of sodium bromide.

Hetherington (1946) found that 500 gamma per ml. of medium was required to obtain growth of brain tissue equivalent to control cultures containing embryonic extract.

23. *Biotin:* Hamilton and Plotz (1942) showed that biotin in concentrations of 1/6 to 5/6 of a gamma markedly improved the growth of embryonic chick and mouse explants, particularly those containing nerve cells. Burt (1943) found that 1/3 of a gamma of biotin added to the medium used for the cultivation of spinal ganglia did not enhance growth as compared with untreated controls. She concluded that biotin was not a promising drug in relation to the stimulation of the regeneration of nervous tissue. Fisher in 1943, using biotin deficient rats, and Lazere, Thompson, and Hines (1943) failed to obtain a significant increase in the rate of regeneration of crushed sciatic nerve with the administration of biotin in excess to the normal intake.

In our own experience, we were able to confirm Hetherington's observation that 5 gamma per ml. prevented the disorganization of nerve cultures for a period of 12 days in unreplenished media. No injury was observed at various dosages up to the limit of saturation of embryonic extract. This gave a final

concentration in the culture medium of 186 gamma per ml. When crystals were incorporated directly into the clot, total inhibition was observed in the presence of undissolved crystals.

24. *Folic Acid:* This substance is of special interest because of its purported noxious effects on nervous tissue in patients suffering from pernicious anemia. Bethell and Sturgis (1948) conclude that the inability of patients with pernicious anemia to utilize folic acid effectively may contribute to neural abnormalities. Until further clarification is obtained regarding this exceedingly knotty problem, many clinical workers follow the opinion expressed by Ross, Belding, and Paegel (1948) that ". . . no patient with pernicious anemia should be treated with folic acid alone." The possible role of contaminating impurities in folic acid preparations requires further elucidation.

Experimental studies based on normal animals agree in concluding that both in animals and in man, pteroylglutamic acid and its relatives are non-toxic. Harned *et al.*, (1946). Sargent (1947) gives the LD_{50} for mice as 600, for rats, 500, for rabbits, 410, and for guinea pigs, 120 mg. per kg. of body weight. Up to 150 mg. of pteroylglutamic acid has been given intramuscularly in man and 500 mg. orally in a single dose without toxic effect. Intravenous administration of 150 mg., however, may result in a shock-like reaction.

Using *in vitro* assay technique, Jacobson and Pomerat (1949) added evidence of the toxicity of various pterine compounds to spinal cord, heart, and spleen explants at dosages relatively much higher than those employed for therapeutic purposes. In the present study, Hetherington's (1946) finding that 1.5 gamma of folic acid appeared to prevent the degeneration of brain cultures was confirmed. The pattern of radiating fiber outgrowth from spinal cord explants resembled that of the control at all concentrations tested including the situation in which crystals (Folvite) were mixed directly in the components of the culture medium. Apparently undamaged nerve and glial processes could be seen in the immediate vicinity of undissolved crystals. Such observations give added reason for doubt that folic acid is directly responsible for degenerative changes manifested in the spinal cord.

25. *Glutamic Acid:* This substance has attracted wide attention at all academic levels because of its capacity to increase motor or psychic activity, particularly with respect to the learning process (Zimmerman and Ross, 1944) and statistically significant improvements in the I.Q. of mentally retarded patients (Zimmerman, Burgermeister, and Putnam, 1947). Greater amelioration is observed in abstract than in motor function. The mechanism of action has excited investigations in the biochemistry of the brain. Weil-Malherbe (1936), for example, demonstrated that 1 (+) — glutamic acid is the only amino acid capable of maintaining oxygen uptake of sliced brain tissue. Nachmansohn, *et al.* (1943) showed that electrical changes during nervous activity are intrinsically linked with the formation and release of acetylcholine. The latter is synthesized by choline acetylase (Nachmansohn and Machado, 1943) which when inactivated by dialysis is reactivated by 1 (+) — glutamic acid (Nachmansohn, John, and Waelsch, 1943). Thus the rate of formation of acetylcholine is increased by the presence of glutamic acid *in vitro* and since acetylcholine is related to nerve activity, the effects of glutamic acid are probably somehow related to this mechanism. Green (1946) considers that ". . . just as myosine is specialized for muscular contraction, rhodopsin or visual purple for photochemical reactions, and hemoglobin for oxygen transfer, so there may be one or more proteins in nerve tissue specialized for reactions which underly the propagation of the nerve impulse."

In tissue cultures, glutamic acid proved to be relatively damaging showing some injury with as little as 6 gamma and total inhibition at 750 gamma per ml. While governing pH was nowhere attempted in the course of our study beyond what is known to be achieved by the efficient buffer mechanisms of the Tyrode-containing plasma clot, it is true that glutamic acid may have exerted its deleterious effect by acidifying the environment of the cells. Nonetheless, within the limits of the conditions as defined in these experiments, the toxicity of glutamic acid closely resembled that found for chloral hydrate and ephedrine.

26. *Para-aminobenzoic Acid:* The importance of this vitamin as an essential nutrient in synthetic media for microorganisms and its interesting capacity to antagonize sulfonamides led us to attempt some observations on its possible role as a stimulant for the growth of nerve fibers. With the end points at our disposal, this attack, however, must await the elaboration of more satisfactory techniques before quantitative data can be assembled. Like folic acid, no evidence of injury was observed in standard preparations incubated for a 48-hour period. When 15 mg. per ml. of crystalline material was incorporated in the medium, undissolved crystals could be seen among the fibers.

Discussion

The evaluation of drug toxicity by means of tissue culture techniques is open to the criticism that the conditions obtained in such preparations are fundamentally different than those of the *in vivo* experiment. For example, in hanging-drop slides without a fluid phase, drugs are directly incorporated in the clotted medium. Under such conditions, diffusion rates may be altered in such a way that far less satisfactory contact may be achieved between the test agent and the cells. A more stringent test might be obtained by growing the cells directly upon glass or cellophane without benefit of a plasma clot. At any rate, under conditions of tissue culture, as pointed out in the introduction to this paper, toxicity is evaluated without the benefit of the specialized detoxifying and eliminating agencies—liver and kidney—of the whole organism. No attempt has been made within the confines of the present study to examine the probable recovery of the damaged elements by means of washing and the replacing of nutrients. The objection may be raised that primary explants do not represent stable systems such as those established in long-time cultures. While the technique of continuous culture has proven to be of undispensable value in the design of certain investigations, it must not be concluded that reliable scientific observations cannot be made with the infinitely simpler technique of the primary explant in a hanging-drop preparation. This is not to say, however, that a vast body of pharmacological data on,

particularly, human cells is not accessible to the cell physiologist with the elaborate procedures now available for long-time survival of cells *in vitro*. Indeed, studies on the antagonistic action of drugs, the minimal dose for reversible injury, the loci of intracellular disturbances, and many other questions invite the experimentalist to utilize the newer knowledge of *in vitro* procedures.

More serious objection to the method presented lies in the fact that no attempt was made to regulate the pH and osmotic pressure changes induced in the culture medium by the drugs under study. Simple hanging-drop preparations not requiring a fluid phase offer little opportunity to make such adjustments. The introduction of phosphate buffers in plasma clots leads to a precipitation of calcium. Various other devices aimed at rectifying imbalances in tissue culture tests other than gassing with CO_2 invariably bring with them complications which produce alterations in the physico-chemical properties of the clot whose effect on cellular migration and multiplication cannot in themselves be easily controlled.

In summary, it may be said that, in common with all other attempts to evaluate the action of chemicals on living systems, caution must be exercised in the interpretation of results. Data must be examined in the light of the phrase "under the conditions of the experiment." With this in mind, the results herein offered demonstrate a direct, rapid, simple, inexpensive, challenging method for the analysis of various chemicals upon nervous and allied tissues.

The following conclusions emerge from the study of 23 substances in contact with explants of chick embryo spinal cords based upon the analysis of more than 8,000 cultures. In Table III, drugs have been grouped to show their relative toxicity *in vitro*.

Group A, comprising strychnine, morphine, atropine, and lobeline were characterized by producing injury over a very short range and being lethal at minute concentrations. While the drugs in Group B, epinephrine, cocaine, benzedrine, glutamic acid, chloral hydrate, ephedrine, nicotine, and camphor tetrazol, showed injurious effects in some instances at doses as low as some members of Group A, the minimal inhibitory

dose (M.I.D.) was generally higher and the range over which injury was demonstrable was much greater. The tendency to show damage over a wide range of concentrations was especially characteristic of paraldehyde, caffeine alkaloid and coramine (Group C). The members of Group D, metrazol, sodium bromide, and nicotinamide produced deleterious effects between 2.5 mg. and 25 mg. per ml. Picrotoxin and biotin were segregated in Group E as showing only slight toxicity. With picrotoxin, outgrowth rated at one plus was observed even in the presence of undissolved crystals while biotin was tolerated at the highest concentrations achieved in solution but the direct addition of crystals did produce inhibitory effects. Group F includes folic acid and para-aminobenzoic acid which were found non-toxic even when undissolved crystals were present in the clotted medium. Both protamine zinc and crystalline insulin showed no injury after 24 hours in the presence of 10 units per ml. but some damage was observed at the end of 48 hours. Barbiturate compounds were not studied with respect to the lowest dose producing damaging effects (L.I.D.), and reference to the total inhibitory dose was drawn from a previous publication. Clinically useful barbiturates probably could be added to Group D.

Substances studied in relation to their *in vitro* toxicity when translated into molal concentrations (Table IV) were found to exhibit the same serial order. This is interpreted as a fortuitous circumstance. It is notable, however, that not only did the most toxic members (Group A, Table III) retain their position but that related sympathicomimetic amines showed similar toxic properties.

Calculations were based on the molecular weight of the free base in the case of strychnine, atropine, cocaine, and morphine. Substances presumably exerting their effects on surface patches such as strychnine, atropine, morphine, and lobeline were found to show the highest toxicity in terms of molal values. The figure also serves to demonstrate again the relatively low toxicity of seconal, metrazol, amytal, pentozarbital, sodium bromide, and nicotinamide as measured by the tissue culture test. The problem of molecular size, configuration,

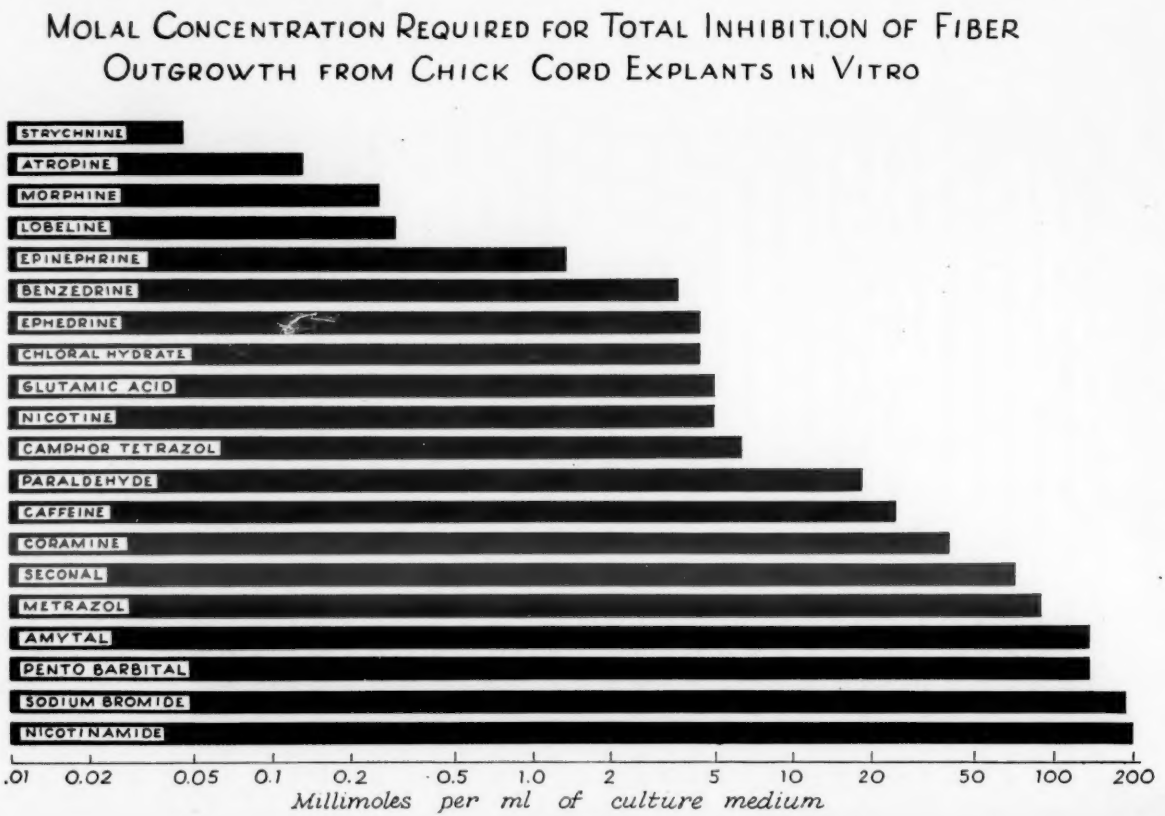

TABLE IV

Molal concentration for total inhibition of outgrowth from spinal cord explants in tissue culture

and reactive groups in relation to toxic effects constitutes an important challenge for further studies.

Verne (1948) has compared toxicity *in vivo* with that found by tissue culture methods by direct weight ratios. Table V represents such comparisons for the substances used in our series. For example, the fatal dose for paraldehyde in man is 100 gms. On the basis of a 75 kg. man this can be expressed as 1:750. In tissue cultures 2.5 mg. per ml. of medium was required for total inhibition or 1:400. The lethal effects, therefore, may be considered as the ratio 1:750 to 1:400 or, 7.87. In other words, on a relative basis nearly twice as much paraldehyde is required to produce the end point described for the *in vitro* test as that for the whole organism. Using the same method of calculation, epinephrine with a lethal dose for of 10 mg. (1:7,500,000) and a MID of 0.25 mg. (1:4000) the ratio is 1,875. The same ratio was obtained for metrazol. These results show that nicotine, metrazol and epinephrine must be used for the tissue culture end-point at a concentration of more than 1,500 times that which would be lethal for man. One must recall the caution sounded in the beginning of this discussion when attempting to interpret the data in this table.

Obviously, man is probably the most unreliable species with which to establish correlations for toxicity studies. For example, our calculations for caffeine poisoning are based on a dosage of at least 10 gm. per 75 kg. of body weight. Not only is variation an important source of error but authentic records in human toxicology are difficult to obtain. Admittedly, our report would have been considerably improved by the inclusion of a table of LD_{50} values for common laboratory animals, particularly the hen. It might be argued further that an ideal comparison of the tissue culture work should have been based on the toxic dose in relation to the development of the chick embryo. It is hoped, nonetheless, that the comparison between the human lethal dose and the tissue culture test will have some clinical value.

The fact that much more material is needed to poison the "free" nerve cells of the tissue culture is seen by the positive ratios shown in Table V in comparison with the lethal amount for man. This is not believed to be due merely to fundamental

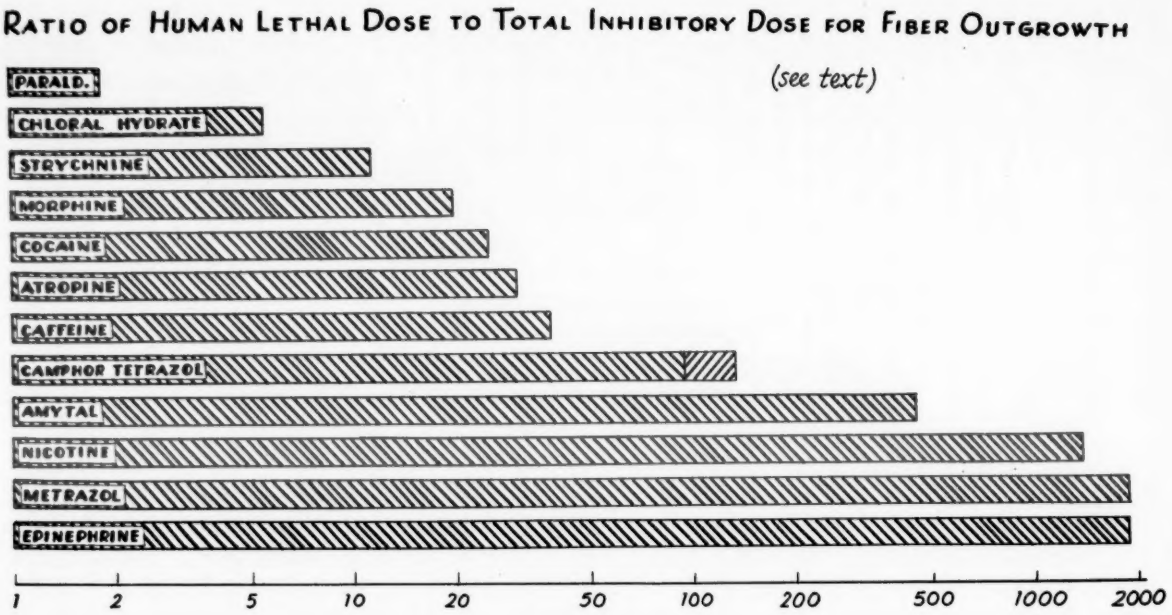

TABLE V

Ratio of toxicity *in vivo* vs. *in vitro*. (See explanation in text.)

differences between species, age of the cells or the opportunities for contact between the noxious agent and the protoplasm. Under *in vitro* conditions the cells are much more nearly independent entities whereas in the individual, division of labor has submitted them to various dependencies upon their neighbors. Toxicity in the *in vitro* test is based upon the death of all cells whereas lethal effects in the whole organism may result from interference with any one of several interdependent functional chains in the homiostatic complex (c.f. Cannon, 1932). The traditional pharmacologist may assert vigorously that the niceties of graphical records of the type obtained with leech muscle will never be displaced by the crudity of the tissue culture method as evidenced by the relatively high values reported as MID's in this report. Our data refer to injury and death of the relatively isolated cell system and in this form their chief merit lies in an opportunity to investigate various cellular sensitivities in order to obtain a picture of the mosaic of specialization in the organism.

Beyond the objective of "lethal topographical relations," graded effects in the function of isolated tissues suggests a valuable new tool for the study of pharmacodynamic action. Thus, Pogogeff and Murray (1944) examined the action of ryanodine with tissue culture methods because under these conditions skeletal muscles maintain spontaneous, rhythmic contraction for long periods in complete isolation from nervous structures. Using the techniques of phase-contrast photomicrography and particularly cinematography Lewis, Pomerat, and Ezell (1949) have reported on the activity of the undulating membrane with special reference to pinocytosis and migration, as well as the movements and changes in the form of mitochondria and nucleoli of the human epidermus *in vitro*. These are but a few of the dynamic end-points which the cytologist may contribute to pharmacological assays. Work in progress using time-lapse photography indicates that "beadings" on outgrowing nerve fibers may show progressive changes in size and position and migration of granules in the axioplasm. Changes in the behavior of mitochondria occur in both the varicosities along the neurite and in the growth cones. Modification in the pattern of formed bodies particularly of micellar

organization, using the technique described by Porter, Claude, and Fullam (1945) for electron microscopy of cells in tissue culture, might yield important data. The method of ultraviolet photomicrography (Hibbard and Lavin, 1945; Lavin, 1946) may also prove very useful in the study of protein synthesis. The student of cell physiology, having before him Quastel's (1943) survey of the effect of narcotics on the respiration of nervous tissue or Lorente de No's (1947) magnificent monograph on nerve physiology, might discover new, delicate cellular end-points. Thus, greater use may be made of recent techniques for the study of living cells in order to establish a closer correlation between structural organizations and the morphological alterations resulting from the action of chemical substances.

Conclusions

1. The toxicity of twenty-three substances known to influence the activity of the nervous system have been studied with the use of more than 8,000 hanging-drop preparations of 9-day chick spinal cord explants observed for a period of 48 hours.

2. Six groups were established in order of toxicity and range of effect for the compounds studied, from those causing total inhibition of outgrowth at as little as 16 gamma per ml. (strychnine) to those proving completely non-toxic even when undissolved crystals were present in the culture medium (folic acid and para-aminobenzoic acid).

3. The serial order of injury was maintained when lethal dosages were plotted as molal concentrations.

4. Ratios between the lethal dose for man as compared with the total inhibitory dose for explants in tissue culture showed relatively greater sensitivity for the entire organism than for the "isolated" nerve cell system. The interpretation of this observation is discussed.

5. The present study suggests a means of locating cells showing special vulnerability within the organism for various chemical agents. Opportunities for using tissue culture methods with newer optical techniques in the quest of more sensitive end-points for describing the effects of chemicals on cells are suggested.

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DELAYED ACTION OF URETHANE PRODUCING DIVERSE TUMORS IN MICE

JOHN G. SINCLAIR*

This is a report of preliminary experiments on mice in which tumors were produced by the use of urethane. The microscopic study indicates some very interesting metabolic phenomena. The experiments otherwise are inadequate to establish any relations between sex, age, dosage, time lapse, and incidence.

Dr. M. Guyer of the University of Wisconsin, using urethane as an anesthetic in rats, had noted the similarity of its action to that of colchicine both in increasing susceptibility to X rays and in its interference with the mitotic mechanism. Keeping some of these rats for four months he also noted the appearance of pulmonary tumors. The strain of rats used had not previously shown such tumors. Dr. Guyer sent samples of the tumors for study. We found that they were all similar spherical adenomas of the alveolar tissue clearly defined but not encapsulated. They were frequently related to terminal bronchioles. We could not determine whether they resulted from hypertrophy of cells in situ or were the result of focal mitoses. There is now a large literature on the production of tumors through the application of carcinogenic agents and radiation to the skin of mice. In the present experiment we used urethane partly because of its chemical simplicity, its supposedly rapid elimination as alcohols and because of its common use for therapy in leukemias. We also decided to stay within anesthetic doses and limit the time for development to less than that at which tumors might be expected spontaneously.

Two groups of twenty-four each were taken from the C3H strain of mice in the colony of Dr. Edgar Poth. They had been selected for two years against spontaneous tumors and showed a very low incidence even at relatively old ages. Since females were kept for breeding purpose the mice we received were supposedly males.

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The first group of twenty-four were injected intraperitoneally or subcutaneously with one-half cc. of a 10% solution of urethane in saline. This was repeated twice a week for three weeks. Each mouse received 300 mg. The mice were young and the reactions were bad. Many mice died within a week and only five of this group survived to the end of the five-month interval.

Because of the difficulties with the first group, a second group was given urethane in drinking water as a 1% solution. Over a three-weeks period each mouse imbibed approximately 300 mg. of urethane. There was no sign of anesthesia and no cessation of activity. Fifteen of this group lived to the sixth month when all were sacrificed.

Mice which died during the waiting period were autopsied but no tumors were found during the two months covered by this series. Marrow smears showed a preliminary loss of primitive lymphoid cells with complete recovery in a few weeks. The peripheral blood showed a temporary loss of lymphocytes. Erythrocytes did not seem to be affected though total counts were not made. The lungs of a number of them showed a pneumonic process and considerable increase in leucocytes.

The survivors for the five- to six-months interval all showed tumor growths. Three of the five remaining from the first group had subcutaneous tumors of the chest and neck. The injections had all been given low in the abdomen or back. Two-thirds of the fifteen left from the second group also showed subcutaneous tumors. They were found in the scrotal region of two, inguinal region of one, while the others had tumors of the axillary, cervical and salivary regions and the back of the neck. All of these mice had pulmonary tumors with a total of one to seven per mouse.

At the time of autopsy it did not occur to us to check the sex of each mouse and the sex organs were not studied in all cases. We know that there were at least two females. There had been no signs of sexual activity during the five-month interval. The testes of two were examined and a peculiar situation was found (Pl. 1, fig. J). Mitotic activity was in full blast and primary spermatocytes filled the tubules. Maturation must have been very rare because only a few sperma-

tozoa were found and there were none in the epididymis. The animals had grown considerably so that mitosis must have been generally normal.

Description of the Tumors

The subcutaneous tumors are histologically of two types. The one large lobulated tumor from the submaxillary region is a mixture based probably on the thyroid and salivary glands. The others are all variations of an adenoma probably of mammary tissue. I have hesitated at this diagnosis because some of these I know were present in male mice.

The mammary type tumors were of several sizes when sectioned. They were alike in that they shelled out from the subcutaneous tissue easily and showed no very evident connections with the skin (Fig. D-6 may be the solid remnant of a duct). They did not show extensions to adjacent tissues and might be classed as adenomas. Sizes ranged from five to fifteen millimeters in diameter. The smaller tumors, 3–5 mm., are non-secreting tubuloacinar masses with very little stroma. The medium tumors, 5–8 mm., are more follicular and contain deposits which might be secretory. Close observation discloses three types of follicle. One (Fig. D, 3–4–5) is clearly secretory and the form of the cells suggests the mammary gland. Behind this is a periacinar line of lymphoid cells in a clear space. A larger follicular space is found to have an endothelial lining often pressed close against a complete row of small cell clusters (Fig. D-C, F-13). Numerous dilated venous spaces also simulate such follicles (Fig. F-14). The largest tumors, 8–15 mm., were hemorrhagic in parts and contained necrotic areas. They were surrounded by thick palisaded walls without the familiar perialveolar lymph cells. On the margins, however, the earlier stages were found.

The mixed tumor of the salivary region is mostly a mass of acinar tissue with mucous characteristics (Fig. F). Ducts seem to be missing. However, some cystic cavities, with stratified squamous walls and filled with pus, might be the remains of such ducts (Fig. G). One-half of the tumor is a mixture in which the mucous central cells are more or less coated by a densely set chromatic epithelium forming follicles (Fig. F,

9–11). Some of these are irregular and some are spheroidal. The spheroidal follicles may contain a translucent acidophil colloid with the characteristics of thyroglobulin. The margins are characteristically vacuolated opposite the cells (Fig. F-12).

The pulmonary tumors likewise were a group with common characteristics. Each is spherical, either subpleural or deeper in position. It is not encapsulated but is sharply defined. They range from one to two millimeters in diameter and in number from one to seven per lung. They vary in size and in differentiation in the same lung and apparently did not start simultaneously. Some are associated with terminal bronchioles but they are certainly not formed by proliferation of bronchiolar epithelium. When they form in a bronchiole the epithelium becomes stratified and densely basophil but it remains in the duct (Fig. I). The usual tumor maintains the architecture of the alveolar walls with much increased thickness because of larger cells and a consequently decreased lumen (Fig. H). The staining reaction also is basophil in contrast to surrounding alveolar walls. Several things are noteworthy. Within the tumor the surface is covered by a tall epithelium while the surface of the normal alveolus is supposedly histiocytic. Examining the lumen of the tumor spaces there are seen to be a large number of clear cells shed from the surface. Tracing the tumor to its margins the surface cells seem to be continuous with deep cells rather than superficial cells of the alveoli. The mitotic rate is not very high.

The lymphoid tissue is of special interest because it shows a great hypertrophy and a peculiar distribution. There is no general lymphocytosis nor invasion of all tissues. Muscles and muscular organs, skin, pancreas and adrenals are free from invasion. On the other hand, there is a general hypertrophy of intestinal patches until they stand out like nodules on the external surface. Considerable increases appear in the whole mucosa from the pharynx downward. The spleen is enlarged and the number of giant cells greatly increased. Specific invasions are seen under the peritoneum of the liver (Fig. B-1) and occasional giant cells are seen deeper in the liver (Fig. B-2). These are in contrast with the macrophages and their yellow granules seen among the lymphocytic cells. The

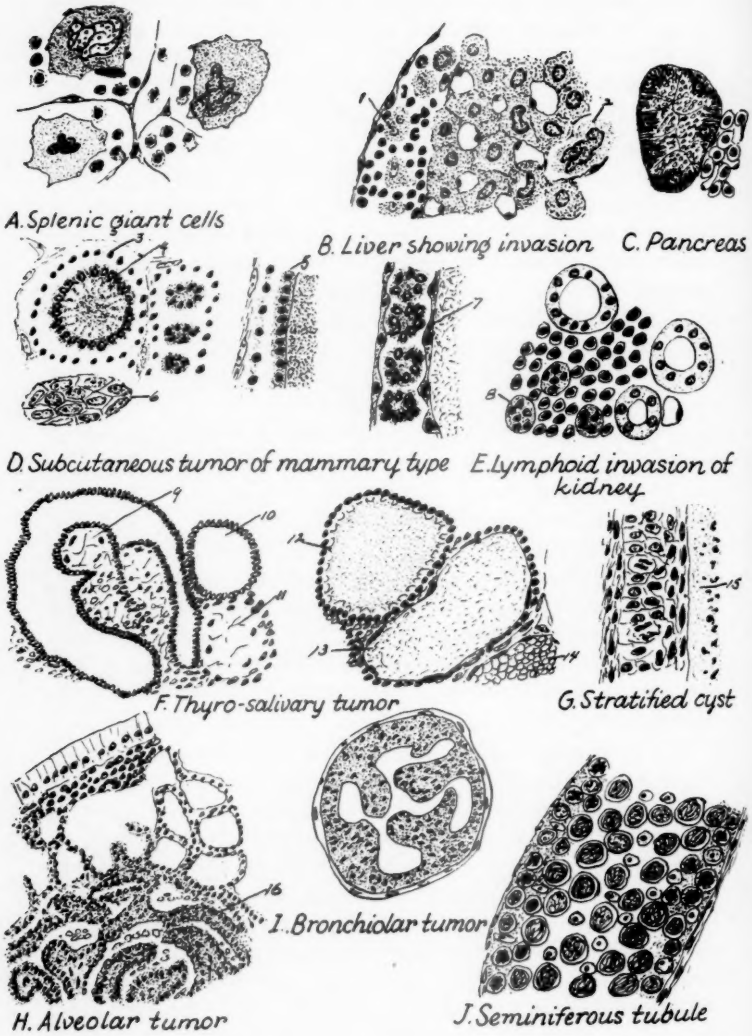

PLATE 1. Details of the varieties of tumors produced by urethane in mice.

kidney in most cases also shows a radial invasion of medium-sized lymph cells together with an atrophy of tubules along that radius (Fig. E-8). These columns occur at widely separated intervals, most of the kidney being quite normal.

Discussion and Summary

Urethane, according to Dr. Guyer, has a more severe action on mitosis than has colchicine. Mitosis is abolished for a short interval. No one has followed the aftermath analytically but there is no suggestion of wholesale cell mutations. The effects seem to be completely obliterated after a short time. The suppression of lymphocytes is presumably caused by cell destruction in addition to the blocking of mitotic lymphoblasts very much like the action of X rays. Therapeutic results in the leukemias have been temporary. No one seems to have reported the lymphocytic rebound seen in all of these otherwise normal mice after urethane treatment.

All of the tumors studied are adenomatous. The rates of mitosis in the subcutaneous tumors were very high at the time we took them so that a carcinomatous change is occurring. This is not true in the pulmonary tumors. Here the size of each is small but they are multiple. The change in cell size to about four times the parent cell, the change to basophil stain and the sharp delineation of these tumors without encapsulation raises a problem of local change in cell metabolism that is hard to understand. The fact that these tumors appear in the lungs first and yet remain smaller than the subcutaneous tumors is also a puzzle.

It is our purpose to pursue the question of the genesis of urethane induced tumors at greater length. The metabolism of this substance is little known but it cannot be as simple as is commonly supposed.

CORRRELATION STUDIES ON PROSTATIC FLUID, PROSTATIC TISSUE AND THE TESTIS WITH HISTOPATHOLOGICAL, PAPANICOLAOU SMEAR AND TISSUE CULTURE TECHNIQUE*

R. E. Cone, C. A. Hooks, C. M. Pomerat, and G. Rose

Screening experiments have demonstrated the desirability and feasibility of attempting to correlate data on cells, particularly in malignant processes, involving the male reproductive tract. Study material includes:

(1) Prostatic secretion smeared and stained by Papanicolaou and related methods.
(2) Tissue cultures of cells in prostatic fluid.
(3) Histopathological preparations of prostatic tissue.
(4) Tissue cultures of prostatic explants.
(5) Histopathological preparation of testicular tissue.
(6) Tissue cultures of testis.

Since the smear technique for the study of cells in prostatic secretions has been clearly described in recent papers (Herbert, 1947; Albers, McDonald, and Thompson, 1949) the present report will only outline some of the methods being employed for the cultivation of cells from prostatic fluid, the prostate and testis *in vitro*.

While simple hanging-drop preparations are ideal for staining and storage in permanent collections, rich out-growth of cells from adult human material is not generally obtained with this method. It has been found, however, that the addition of one or two drops of human ascitic fluid to the surface of clotted medium may enhance growth in such preparations (Lewis, Pomerat, and Ezell, 1949). The Maximow flying-drop technique is particularly well adapted for this management of the medium. Carrel flask and Gey's roller-tube methods have proven especially successful when a supernatant nutrient consisting of approximately 50% chick embryonic extract has

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been utilized. Illustrations of typical results obtained with our techniques are presented. These were not all based upon the study of materials from one individual nor is any attempt made in this communication to predict what conclusions ultimately may be drawn from this work. Correlative cytological studies require experience which can only be obtained by an intensive study of a large quantity of selected material. The results may provide a more fundamental basis for evaluation and clinical management of urological lesions. At present the most accessible materials are malignant neoplasms of the prostate and bladder, and testis biopsy in male infertility.

Figure 1 is a photomicrograph of a prostatic smear stained with Papanicolaou's method while figure 2 represents a Carrel flask culture of the same material after 3 days of incubation at 37° C. Figures 3 and 4 show an epithelial sheet grown from a specimen of adenocarcinoma of the prostate while cultures of the testis of the same patient are represented in figures 5 and 6. The epithelium shown at high magnification in figure 4 is believed to be the malignant agent.

The arrow in figure 7 indicates the presence of an interstitial cell of the testis, shown at much higher magnification in figure 8, which contained 4 nuclei. Such quadri-nucleate cells have been frequently encountered in the testes of patients with carcinoma of the prostate but adequate control material is lacking.

Figure 7 shows an isolated seminiferous tubule from which interstitial cells are seen to be migrating. These cells were photographed in the living state while those in figure 8, from another culture at somewhat higher magnification, were stained. Multi-nucleation and nuclear irregularities are common.

Beyond the opportunity to correlate cytological data obtained by the methods suggested in these photographs, additional information may be obtained by the systematic addition of various hormones in the culture medium. A crude approach to this technique has been suggested in a study on ascitic fluid by Ivers, Neidhart, and Pomerat (1948). Moreover, environmental influences such as temperature variation may be used in these studies particularly for the testis under highly controllable conditions. Implantation into the anterior chamber

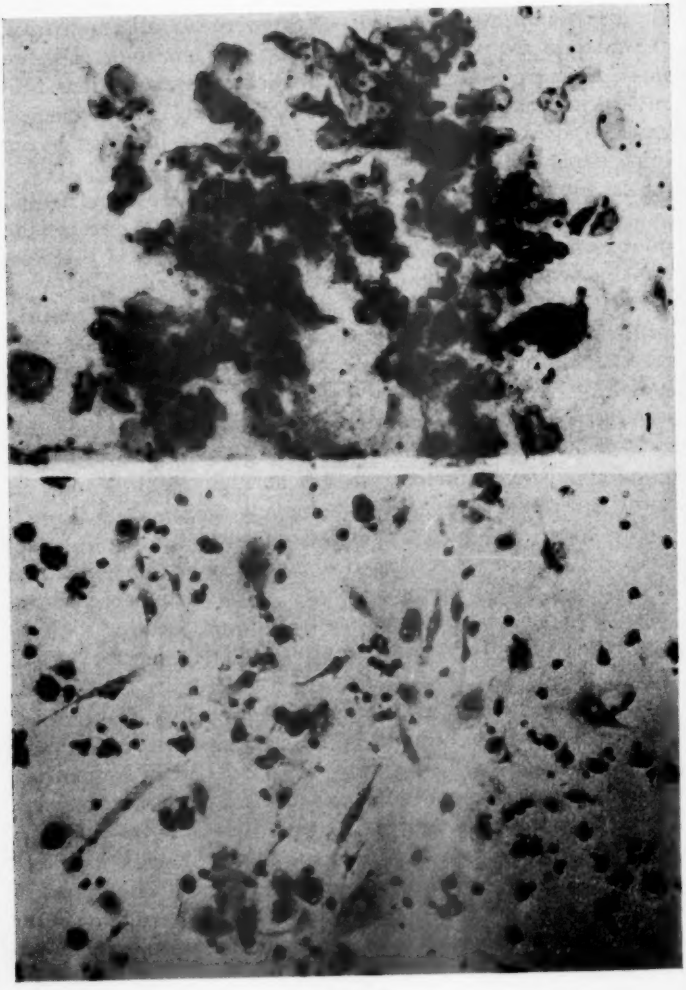

PLATE I

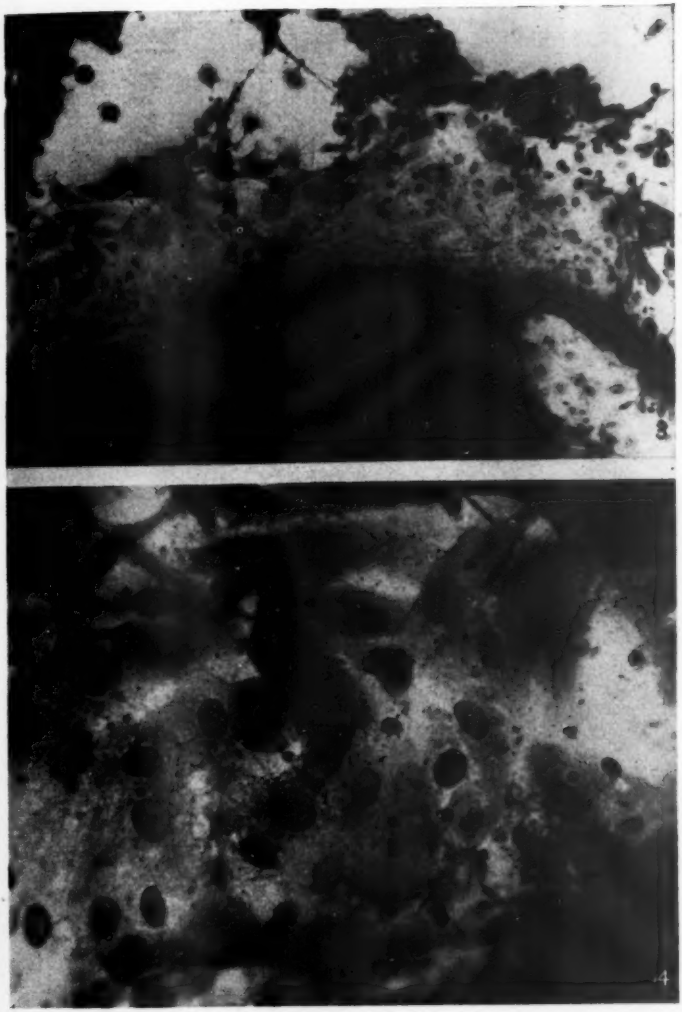

PLATE II

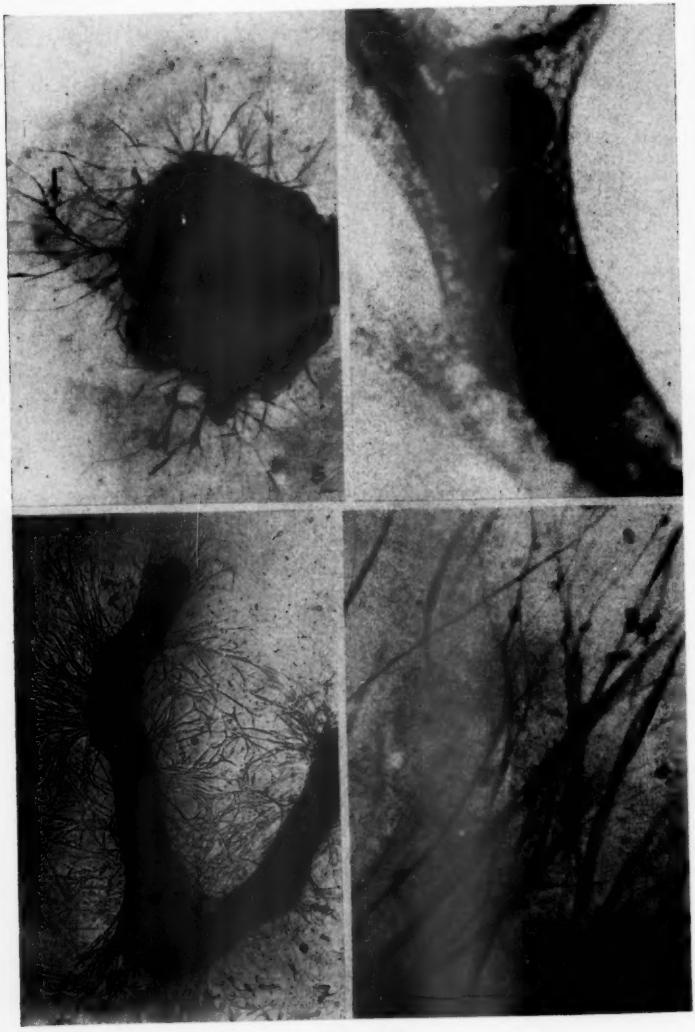

PLATE III

of the guinea pig's eye (Greene, 1947) may serve as a very useful check on events taking place in malignant tissue cultivated under experimental conditions.

Similar correlative studies between the cytological features observed in the direct smears as contrasted to the transformations which can be seen *in vitro* for exfoliated cells from the urine, vagina, lung, and stomach also invite attention.

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A TECHNIQUE FOR THE HYPOPHYSECTOMY OF THE SNAKE*

GLENN A. DRAGER

The literature contains but few reports on the hypophysis of reptiles and even fewer reports of methods of hypophysectomy. Schaefer (1933) and Rahn (1941) have reported certain effects of hypophysectomy of snakes but do not describe the method of hypophysectomy in detail. Regardless of the simplicity of the perfected method employed in hypophysectomy it is often time consuming to work out each detail of a procedure. Only can adequate descriptions of techniques alleviate such unnecessary expenditure of time and waste of animals. In conjunction with other investigations of the neuroendocrinology of the snake the following method of hypophysectomy was adopted.

This method is particularly designed for the hypophysectomy of the Tropical Indigo snake, but with modification the method may be adapted to any species of snake.

Anesthesia. Nembutal (60 mg. per cc. 10% alcohol solution) was found to be the most easily used anesthetic. The effective dose is 18 to 20 mg. per kilogram body weight administered intraperitoneally. In the beginning of the experiment 25 to 30 mg. per kg. (usual dosage for mammals) was given. Some animals receiving this amount of the drug remained "out" for nearly a week. Approximately 30 minutes is required following injection of the nembutal before the animal is completely anesthetized.

Procedure. The animal is placed ventral side up on a table and the head secured under rubber bands retained with thumb tacks. The lower jaw is depressed and held in place with a hemostat tied back by means of a string and tack (fig. 1). The oral mucosa is incised and retracted. The sphenoid bone is then cleansed of tissue. Often at this stage a small amount of bleeding may occur which can be stopped by swabbing the

*From the Department of Anatomy, The University of Texas Medical Branch, Galveston. Received for publication, June 4, 1949.

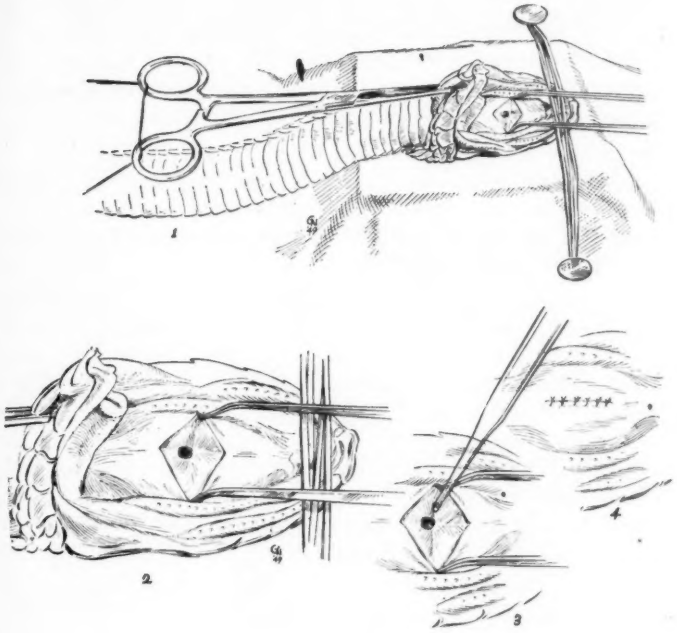

FIGURE 1

area with adrenalin chloride solution or by a cautery. As the bone is cleansed a slightly darkened area of the sphenoid becomes visible. This area outlines the sella turcica and appears darker because of the numerous melanophores in the hypophyseal capsule. A hole is drilled through the sphenoid, by means of a dental drill equipped with an angle handpiece and a No. 8 burr until the pituitary gland is reached. The hole is then enlarged with a No. 39 burr until nearly all of the pars distalis is exposed (fig. 2). The capsule is incised and the hypophysis removed with a suction cannula. The empty sella is shown in figure 3. Care must be taken to remove all of the posterior lobe as the anterior lobe often breaks away from the pars nervosa. Following removal of the gland there

usually is a slight amount of bleeding which will stop if a small cotton pellet (No. 4) is inserted into the empty sella. After a few minutes the cotton pellet is removed and the sella is then packed with fibrin foam to safeguard against additional hemorrhage. The mucosa is then sutured with cotton thread (fig. 4).

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SERUM PARA-AMINOSALICYLIC ACID (PAS) FOLLOWING ORAL INGESTION IN CHILDREN

LARS SÖDERHJELM*

Since the initial study by Lehmann in 1946 many favorable reports have been published concerning the effectiveness of p-aminosalicylic acid (PAS) in tuberculosis in man. No definite agreement, however, as to the possible effective dosage has been reached. The decision to use PAS in conjunction with streptomycin in the treatment of four children with tuberculosis afforded an opportunity to study the relationship between dosage and serum values in children.

In vitro studies by Sievers (1946) revealed that a concentration of PAS of 0.15 mg. per cent was sufficient to inhibit the growth of tubercle bacilli. In 1949, however, he reported that some strains of M. tuberculasis proved to be resistant at a concentration of 0.15 mg. per cent, but all the strains studied were sensitive to 1.5 mg. per cent. Some of the organisms examined were cultured from patients who had received the drug for prolonged periods.

Observations with experimental animals by various workers (Lehmann, 1946; Paraf, Desbordes, Henry, and Paraf, 1948; Ragaz, 1948; Venkatamaran, Venkatamaran, and Lewis, 1948; Way, Smith, Howie, Weiss, and Swanson, 1948) regarding the absorption and blood concentrations of the drug in all instances reveal evidence of rapid absorption after oral ingestion with the attainment of maximum blood levels in 30 to 60 minutes, and almost complete excretion in the urine within 3 to 6 hours.

In man Lehmann (1946) recommended that PAS be given every 4 hours in order to maintain a blood concentration between 3 and 6 mg. per cent. Way and his collaborators (1948) studied the absorption and excretion of PAS and found that after 4 g PAS *per os* in human subjects the maximum blood

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level was reached in 1–2 hours, and by 7 hours 85 per cent had been excreted in the urine. Paraf and co-workers found (1948b) in adults, given 4 g by mouth, that in 30 to 60 minutes the blood levels were 4.8 to 9.4 mg. per cent, and within 6 hours the drug had disappeared from the blood stream. In therapeutic studies by Swedish investigators (Alin and Difs, 1947, Carstensen and Sjölin, 1948; Carstensen and Söderhjelm, 1948; Vallentin, 1946) the dosage of PAS in adults generally is 10 to 18 g per day divided into 5 equal doses. Paraf (1948a) and Nagley and Logg (1949) gave much larger amounts, but lower dosage is used by Swiss workers (Hug, Moeschlin, and Tanner, 1949; Steinlin and Wilhelmi, 1948) in that the drug is given only 4 or 5 days of the week.

Material and Methods

Pertinent information concerning the children in this study are listed in Table 1.

TABLE 1

Data Concerning Four Children With Tuberculosis Used in This Study

Case No.	Sex	Age (years)	Body weight (kg)	Diagnosis	Daily dosage of PAS
1.	M	12	27	Tbc. meningitis	1.7 g 5 times daily
2.	F	¾	8	" "	0.5 g " " "
3.	M	4	16	" "	1.0 g " " "
4.	F	4	17	" spondylitis	1.0 g " " "

Both PAS and the sodium salt were used. The p-aminosalicylic acid (obtained from Hexagon laboratories) was administered in capsules or as a 25 per cent suspension in water, and the sodium p-aminosalicylate dihydrate* was given as a 10 per cent aqueous solution. The drug was given during the day at 4 hour intervals in five equal doses. The total dosage was 0.3 g per kg. body weight per day, which is higher than the dose of 0.2 g per kg. recommended by the Tuberculosis Study Section of the U. S. Public Health Service (1949).

*Supplied through the courtesy of Merck and Company.

The method of Klyne and Newhouse (1948) was used for determining PAS on aliquots of 0.5 ml. serum. More accurate values are attained with the use of serum than whole blood in this method. After precipitation of the portein of the serum with p-toluene sulfonic acid, the filtrate was buffered and Ehrlich's reagent added. Streptomycin does not interfere with the reaction according to the authors. The color was read in a Klett-Summerson photoelectric colorimeter using a blue filter (no. 42). There was good agreement in duplicate samples. This method determines only the free PAS in the serum and not the portion which is bound to protein. It is essential that the standard solution of PAS be fresh. It was found that the serum, however, may be stored in a refrigerator several days without changing the PAS values.

When samples of blood were obtained at variable intervals during the day and night, the values showed a wide variability. Hence, the serum levels of PAS were determined subsequently at exactly recorded intervals following the ingestion of the drug.

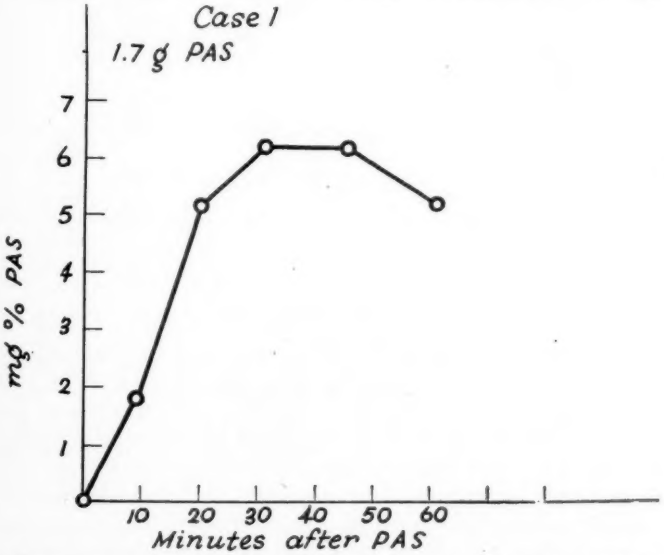

FIG. 1. Serum levels of PAS in Case 1 following oral ingestion of 1.7 g. PAS (= 0.06 g. per kg. body weight).

A portion of the results are summarized in Figures 1–5. The values plotted represent about one-half of the total determinations. The other values obtained were in agreement with those shown in the graphs.

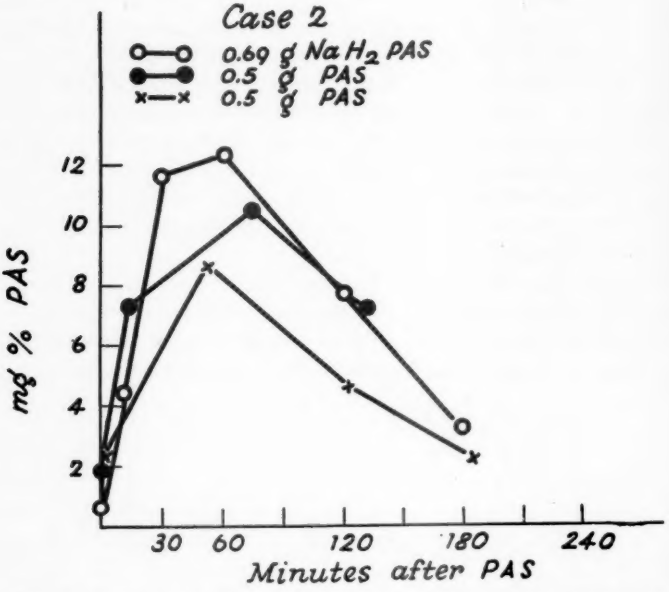

FIG. 2. Serum levels of PAS (Case 2) following oral ingestion of PAS on two occasions and corresponding amount of sodium p-aminosalicylate dihydrate.

DISCUSSION

The initial determination for serum level of PAS usually was made 4 hours after the preceding dose of PAS (0.06 g/kg body weight); hence, the first value for each curve does not start from the zero base line. The rapid rise of the serum level of PAS after oral ingestion is shown in Figure 1; here the determinations were made at intervals of 10 to 15 minutes. Almost identical curves were obtained after PAS and after corresponding amounts of sodium-PAS indicating the same rate or degree of absorption (Figs. 2, 4, and 5). Although values

of 10 to 12 mg. per cent were reached in 30 to 60 minutes, three hours after the drug was given 2–3 mg. per cent was usually present. After four hours (Fig. 2) there was 2–2.5 mg. per cent present; however, these values represent the zero hour in the patients who were receiving PAS regularly. Concentrations in this range are well above the minimum bacteriostatic levels *in vitro*. In a later experiment this same child (Case 2) received a somewhat smaller dose (0.5 g per kg. body weight), administered only 4 times daily as recommended by the Tuberculosis Study Section. As may be seen in Fig. 3, already in three hours there was less than 1 mg. per cent, and in 6 hours only 0.18 mg. per cent at the time the next dose was scheduled to be given. This low level of PAS, however, may be sufficient to prevent the development of resistance to streptomycin as shown *in vitro* by Graessle and Pietrowski (1949).

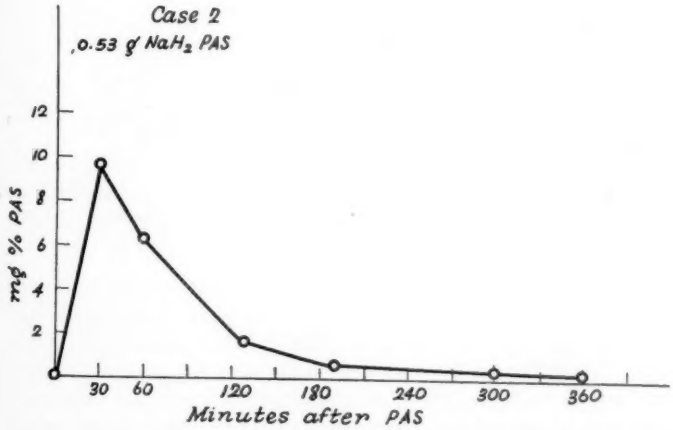

FIG. 3. Serum levels in Case 2 for a period of 6 hours following the oral ingestion of 0.05 g. PAS per kg. body weight.

From the rather sharp decline in all the curves it seems quite clear that there is a rapid excretion of PAS. This observation confirms earlier studies with adult subjects (Way and others, 1948; Ragaz, 1948). As revealed quite significantly

in these studies, in order to ascertain the exact serum levels, it is very important to make the determination not only after a certain dose of PAS, but also at a fixed time interval following administration of the drug.

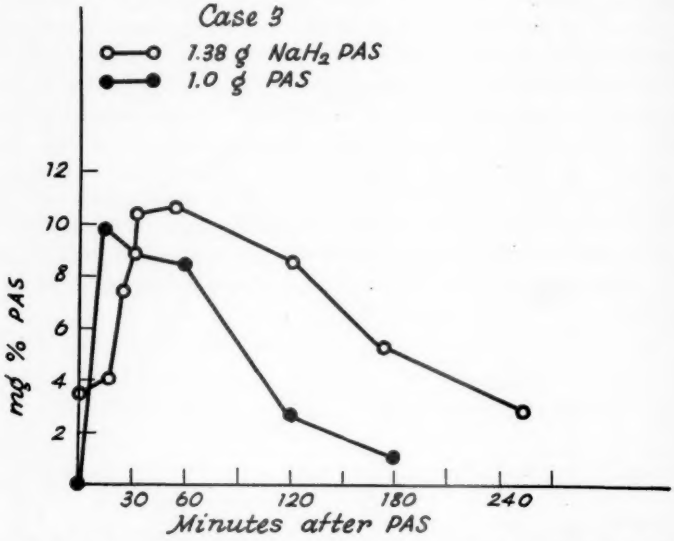

FIG. 4. Serum levels of PAS in Case 3 following oral ingestion of 0.06 g. PAS per kg. body weight and corresponding amount of sodium p-aminosalicylate dihydrate.

The spinal fluid levels of PAS on a number of occasions were studied in Cases 1, 2, and 3. In Case 3 the level was 0.4 mg. per cent 170 minutes after the first dose (0.06 g per kg.). Later during the course of treatment the level was above one mg. per cent on three occasions and 0.6 mg. per cent once. In Cases 1 and 2 four determinations were made on the spinal fluid, and in all instances PAS levels were above 1.5 mg. per cent. On the basis of these studies there seems to be no relationship between serum and spinal fluid levels, the latter being more constant throughout the day.

Therapeutic evaluation of these cases is not possible in this four-month interval of observation. All three children with

meningitis are still living, but in Cases 2 and 3 there is evidence of severe cerebral disturbance. Hearing is completely absent in Case 1, and the spinal fluid shows elevation of protein and pleocytosis. Tubercle bacilli have not been isolated from the spinal fluid since the combination of PAS and streptomycin has been employed. It is not possible to interpret any special change in the conditions of the child with spondylitis. No signs of toxicity such as leucopenia or vomiting occurred in these children.

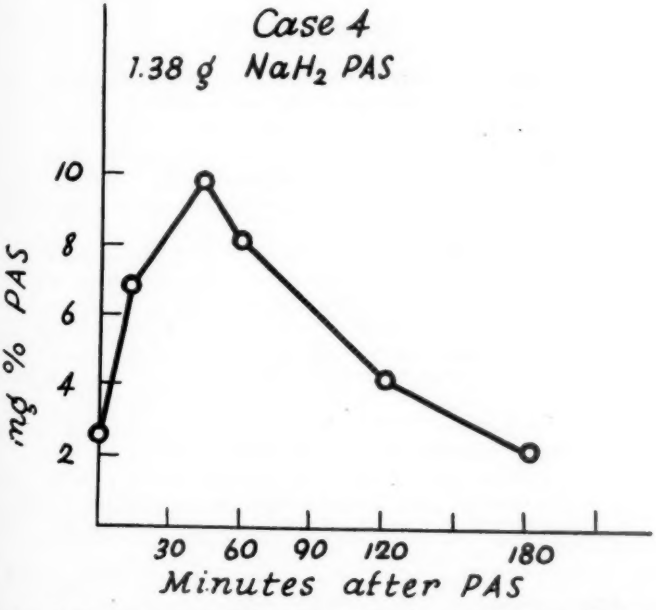

FIG. 5. Serum levels of PAS in Case 4 following oral ingestion of sodium p-aminosalicylate dihydrate corresponding to 0.06 PAS g. per kg. body weight.

SUMMARY

About 130 determinations of the serum level of p-aminosalicylic acid (PAS) have been made in 4 children with tuberculosis receiving combined streptomycin and PAS therapy.

The serum levels increase rapidly following the oral administration of PAS with maximum values in about 60 minutes, then there is a sharp decline, which indicates that elimination readily occurs. There is no apparent difference in serum levels whether PAS or its sodium salt is given.

The total dosage of 0.3 g per kg. body weight per day, given in 5 doses or every 4 hours during the daytime, appears to establish adequate bacteriostatic serum levels, except during the early morning hours because of the omission of the night dose.

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FAILURE TO PROPAGATE HUMAN POLIOMYELITIS VIRUS IN DEVELOPING AVIAN EMBRYOS*

MORRIS POLLARD

When Gard first reported that he was able to propagate a human poliomyelitis virus in the developing chicken embryo, he caused many confirmatory programs to be initiated (Burnet, Stimpert, Kast and Kolmer, Riordan and Sa-Fleitas). It has now become quite apparent that these latter investigators were able to propagate only the agent of murine encephalomyelitis (Theiler's virus, mouse poliomyelitis) in the developing chicken embryo, and their reports and the reports of others indicate that human poliomyelitis virus will not grow in this medium (Dunham and Parker, Riordan and Sa-Fleitas, Powell and Jamieson). Enright and Schultz have reported that an aberrant Lansing-type poliomyelitis virus was propagated in the developing chicken embryo; however, this virus has recently been identified as a strain of murine encephalomyelitis (Frisch and Hehriques).

Since it seemed obvious that the tissues of the developing chicken embryo would not support the growth of human poliomyelitis virus, it was of interest to determine whether avian embryos, other than that of the chicken *(Gallus domesticus)* would provide a proper medium for propagation of this virus. The developing embryos of the turkey *(Meleagris gallopavo)* and the duck *(Anas boschas)*, along with the chicken, were inoculated with virulent poliomyelitis virus suspensions and through manipulations in dosage, route of inoculation, age of embryo, temperature and duration of post-inoculation incubation, some condition was sought by which the virus might be propagated. All experiments have thus far been negative.

The poliomyelitis viruses which were employed in this study were as follows:

1. MEF_1—a Lansing-like strain (Schlesinger *et al.*) kindly supplied by Dr. P. K. Olitsky.
2. Lansing—Standard virus (Armstrong) supplied by Dr. Thomas Francis, Jr.

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3. Mahoney and Rockford—two simian infective strains supplied by Dr. Thomas Francis, Jr.

4. Riley—a virus of recent isolation, infective for cynomolgus monkeys via the oral route. This virus was obtained from Dr. David Bodian.

The avian embryos were purchased from pullorum-free commercial hatcheries. They were incubated at 39° C. until inoculation and thereafter at temperatures such as 35° C., 37° C., and 39° C. Embryos at 4, 6, and 8 days of age were inoculated with poliomyelitis-infected nervous tissue emulsions into the chorio-allantoic sac (Burnet), amniotic sac (Beveridge and Burnet), and yolk sac (Cox). Eleven-day-old embryos were inoculated intravenously according to the technique described by Eichhorn. The egg contents were transferred at intervals of two, five, six, eight, and ten days, through blind passages of five series of embryos. In order to determine whether virus was present in the egg content, emulsions thereof were inoculated intracerebrally into mice (with MEF_1 and Lansing viruses) or similarly into *Macacus mulatta* monkeys with the non-Lansing viruses. The inoculated animals were observed daily for evidence of infection for thirty days following inoculation and any that appeared to be ill, were passaged (brain and cord) to new animals. Histological examinations were made of all tissues, particularly of the central nervous system, for evidence of infection. All surviving animals were challenged intracerebrally with the homologous virus soon after the termination of the thirty-day observation period. Each experiment was repeated at least twice, and in many instances, three or four times.

Results and Discussion

The virus of human poliomyelitis was not propagated in any of the avian embryos used. When chicken and duck embryos were sacrificed on the second day following inoculation with infective MEF_1 virus suspension, the virus could be recovered in mice. All incubation periods longer than two days were uniformly negative for virus. Attempts at alternate egg-mouse-egg-mouse passage with MEF_1 virus (using the two-day incubation period in eggs) failed to result in any adaptation of the

virus to the egg. Chicken embryos were inoculated with anti-chicken serum* plus MEF_1 virus and while a considerable number of embryos died or developed with anomalies, no evidence of virus growth could be demonstrated. On two occasions, goose eggs were inoculated into the amniotic cavity with MEF_1 virus but there was no virus present ten days later when the tissues were harvested and inoculated into mice. Autolyzed MEF_1-infected mouse cords were inoculated into the yolk sac of 8-day-old turkey embryos, in accordance with the idea of Milzer and Byrd that such treatment broadens the animal-infective spectrum. The virus, thus treated, failed to survive for six days in the turkey embryos. The turkey, chicken, and duck embryos which were inoculated intravenously with MEF_1 virus hatched out and appeared to be normal.

All of the mice and monkeys which were inoculated with the embryo emulsions described above were not immune to intracerebral challenge with the homologous virus. Even if the virus had been altered through egg passage, some slight degree of immunity in the inoculated animals should have been induced. The results of these challenge inoculations, therefore, help to confirm the absence of poliomyelitis virus in the original chick embryo material.

It appears, therefore, that with the orthodox techniques which are employed for the propagation of many of the well-known viral agents, human poliomyelitis virus cannot be propagated in the developing embryonic tissues of the chicken, turkey, and duck. While the poliomyelitis virus will survive *in vitro* at proper pH levels and at 11° C. for several weeks (Pollard *et al.*) and for at least one week at 35° C., it appears to deteriorate at a relatively rapid rate in the tissues of avian embryos. We have not yet been able to identify the intolerant mechanism which these avian tissues seem to exert towards the virus.

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PRESENT STATUS OF TREATMENT OF SEVERE BURNS*

T. G. Blocker, Jr.

There has been a tendency within recent years for the local treatment of extensive burns to become more and more simplified with greater emphasis upon measures to combat shock, dehydration, alterations in the circulating blood volume, protein and electrolytic imbalance, anemia, and infection. Away from medical centers, however, there is still too much emphasis upon the use of proprietary preparations, repeated dressings, and useless efforts to promote epithelialization on areas where it should be obvious from the onset of injury that early skin grafting will be required for adequate coverage and to prevent the formation of burn scars and contractures.

Under optimum conditions the average burn patient should be ready for skin grafting within three or four weeks. Any surgeon who is cognizant of the pathological physiology involved can handle burn cases satisfactorily if he has learned the technique of skin grafting with the dermatome. An extensive severe burn is a full-time job for one man, however, and if the general surgeon does not have at his disposal the facilities or personnel necessary for a so-called burn team, he should refer the patient to someone who does. Cosmetic and functional results are dependent upon infinite care and adherence to certain plastic principles which have emerged from observation and treatment of acute burns and old burn deformities. The plastic surgeon will have a much better chance to achieve good results if he knows the overall picture from the early stages of treatment and can plan reconstructive procedures accordingly. It is unfair to him and to the patient to temporize for weeks and months, postponing surgery in instances of extensive third-degree and severe second-degree burns until the individual has developed such a state of malnutrition and "chronic shock" that skin grafting cannot even be at-

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tempted without a long period of intensive preoperative treatment.

Acute extensive burns should be covered with pressure dressings under strict aseptic conditions to avoid contamination of the open wounds by attending personnel. Widespread debridement with evacuation of bleb fluid is contraindicated. Only gross dirt and bits of clothing should be removed, and for this purpose bland soap and water may be applied with cotton pledgets followed by irrigation with copious amounts of saline solution. No chemicals or antiseptics should be employed on burned areas, and the use of sulfonamides has been contraindicated in view of variability of absorption and the possibility of an adverse effect on the urinary output. As an immediate cover any material may be used which will be nonadherent and nonirritating. Rayon wet with normal saline and vaseline gauze are readily available. At the present time we are employing either gauze impregnated with furacin or a wet dressing of 1:4000 zephiran with .5% acetic acid. Although we still employ .2% pyruvic acid in KY jelly as an aid to the separation of the slough in some instances, we have abandoned its use as a routine procedure chiefly because many patients complain of excruciating pain for about 30 minutes after it is applied, even under the influence of opiates. A bulky layer of fluff gauze and mechanics' waste should be applied evenly over the initial covering of the burn with an elastic bandage to hold it firmly in place without interfering with the circulation. Severe burns of the face are similarly treated and we do not hesitate to cover the eyes, after suitable protection, if necessary to secure bandages properly. The patient will experience far less discomfort and run less risk of infection than if hit-or-miss methods are employed. Unless dressings are grossly contaminated or there is evidence of infection which would make advisable the substitution of wet dressings for dry, they should be left in place for ten or twelve days before changing.

The administration of T.A.T. or booster doses of toxoid must never be overlooked, and the patient should be placed upon large doses of penicillin in procaine solution.

At the time of initial examination of the patient an estimate should be made of his requirements for plasma and other intra-

venous fluids for the succeeding 24 hours, provided that he has sustained third-degree burns involving 10% of the total body surface or 20% of lesser degree.[1]

The amount of fluids to be given may be calculated by a number of different methods. The most recent guide to therapy has been formulated by Cope[2] and his associates, working with thiocyanate and radiosodium, on the basis of anticipated tissue space expansion resulting from the collection of edema fluid in the wound area. This amount far exceeds the external loss and is dependent upon a number of variable factors such as location and extent of the burn, gradations in degree of capillary injury, etc. Since the interstitial fluid contains relatively more water and less protein than the circulating plasma, there is hence in the latter an initial concentration of protein which is responsible for rapid dehydration of unburned areas of the body by reason of increase in plasma osmotic pressure. After dilution of plasma occurs, a secondary hemo-concentration is produced by the demands of tissue space expansion as the wound edema continues to increase over a period of 36 or 48 hours. Plasma and fluid therapy during this period must be intensive in order to prevent irreversible changes in unburned tissues of the body and to maintain an adequate circulating blood volume.

Cope recommends 3,000 cc. of fluid daily to replace normal renal excretion and insensible loss, half as isotonic electrolyte intravenously and half as oral fluids or glucose in water by vein. To take care of wound edema for 48 hours he advocates an amount equal to 10% of body weight, supplemented by 1,000 cc. for burns involving 25–35% of body surface to offset external fluid loss, 2,000 cc. for 35–60%, and 3,000 cc. for

[1]Berkow's table of estimating body surface is as follows:

Head—6%	(for children add 12 minus age)
Upper extremities —18%	(for children add half of 12 minus age)
Lower extremities,—38% including the buttocks	(for children subtract 12 minus age)
Trunk, including —38% the neck and genitalia	(for children add half of 12 minus age)

[2]Cope, Oliver, Graham, John B., Moore, Francis D., et al.: The Nature of the Shift of Plasma Protein to the Extra-vascular Space Following Thermal Trauma. *Annls. of Surg.*, Vol. 128, p. 1041, Dec., 1948.

Cope, Oliver, Moore, Francis D.: The Redistribution of Body Water and the Fluid Therapy of the Burned Patient. *Annls. of Surg.*, Vol. 126, p. 1010, Dec., 1947.

60% and over. Two-thirds of this emergency fluid he feels should be administered as plasma, and one-third as isotonic electrolyte with half of the total amount given in the first 12 hours, one-fourth in the next 12, and one-fourth in the second 24 hours. Thus, as an example, a woman weighing 60 kilograms with burns extending over 60% of the body surface would receive during the first 12 hours 3,000 cc. of plasma, 2,250 cc. of isotonic electrolyte, and 750 cc. of oral fluids (or glucose in water by vein). During the next 12 hours she would be given 1,500 cc. plasma, 1,500 cc. of electrolyte solution, and 750 cc. of oral fluids. During the second 24 hours she would have 1,500 cc. of additional plasma, 2,250 cc. of isotonic electrolyte and 1,500 cc. by mouth. Total for the period would be 6,000 cc. of plasma, 6,000 cc. of i–v fluids, and 3,000 cc. of oral fluids.

Other methods of estimation of plasma requirements which have been employed in the past are 50 cc. for each 1% of body surface involved for the first 24 hours and 100 cc. of plasma for each point of elevation of the hematocrit above 45, as recommended by Harkins. In the latter method the hematocrit is taken every 6 hours. If it is rising or shows no change a new calculation is made on the basis of that reading. In medical centers there is often a tendency toward over-treatment with fluids. Proper regard must be taken of the fact that following maximum wound edema resorption of fluid usually begins on the third day with spontaneous diuresis. At this time general and pulmonary circulation may be embarrassed and too great a burden placed upon the kidney if the patient has been the victim of excessive fluid therapy.

Perhaps the simplest method of estimating total fluid requirements in small children is on the basis of 70–90 cc. per pound for a 24 hour period with one-fourth or one-third of the total (in severe shock, even more) given in the form of plasma. With syringe transfusion 10 cc. per pound may be given initially and repeated as required so that approximately half of the estimated plasma is given in the first 6 hours, one-fourth in the second 6 hours, and one-half in the remaining 12. If oral intake is limited, the remaining fluids should be

given in the form of intravenous 5% glucose in water and clyses of normal saline or 2.5% glucose in saline.

Since fluid requirements are at best guess work, it is essential that the surgeon keep a constant check on the patient's progress as judged by his general appearance, evidence of dehydration, pulse, respiration, blood pressure and temperature readings. At the end of 48 hours plasma should be replaced by transfusions of whole blood to relieve anemia, maintain adequate circulating blood volume and prevent nitrogen depletion.

In view of the grave prognosis associated with lower nephron nephrosis as evidenced by oliguria or anuria and elevation of nonprotein nitrogen, it is important to keep a constant check on urinary output from the onset of treatment and to repeat blood chemistry studies at frequent intervals in the more severe burn cases. The best treatment of acute renal insufficiency produced by hypotension and reduction in circulating blood volume we believe to be that of Muirhead and Hill,[3] who limit fluid intake to urinary output plus 800 to 2,000 cc. allowance for insensible water loss. To combat acidosis they advocate 4 gm. of sodium bicarbonate by mouth every 6 hours. A low salt diet is prescribed with at least 1 calorie per cc. of fluid intake. Loss of appetite with subsequent low protein intake is chiefly responsible for negative nitrogen balance in burn cases once the acute stage is passed. Keyser[4] postulates also a period of hormonal imbalance which interferes with nitrogen equilibrium. Clark, Nelson, Lyons,[5] and their coworkers have developed the concept of chronic shock in individuals with severe burns, other unhealed wounds, or long-standing debilitating conditions of one type or another in which anorexia interferes with proper nutrition of the patient. As a result of inadequate diet anemia and hypoproteinemia develop. These may be obscured by lowered circulating blood volume just as the accompanying increase in interstitial fluid

[3]Muirhead, E. E., Hill, J. M.: The Treatment of Acute Renal Insufficiency. *S.G.&O.*, Vol. 87, p. 445, October, 1948.

[4]Keyser, J. W.: Metabolic Study of Burned Cases. *Annls. of Surg.*, Vol. 127, p. 605, 1948.

[5]Clark, John H., Nelson, Woodrow, Lyons, Champ, et al.: Chronic Shock: The Problem of Reduced Blood Volume in the Chronically Ill Patient. *Annls. of Surg.*, Vol. 125, p. 618, May, 1947.

volume may hide to some extent evidence of marked weight loss and decrease in muscle volume. Patients with chronic shock are notoriously poor operative risks. The only satisfactory method of prevention or remedy is the use of repeated transfusions of whole blood and the administration of protein far in excess of ordinary requirements. Amino acids given intravenously are rapidly excreted and cannot be depended upon to provide adequate therapy. In addition they are likely to destroy the veins at the site of injection. We have found most useful in all our burn patients the use of a solution of liver protein hydrolysate, which we administer as a constant drip through a small intranasal plastic tube (purchased from Surprenant Manufacturing Company) into the stomach. The caliber of the tubing is that of an 18-gauge needle, which is fitted into the end of the tube and attached to an ordinary Murphy drip bottle apparatus containing 24 hours' supply of the preparation. The tubing is easily inserted, and as it becomes warm and flexible from the body heat gives no discomfort to the patient even while he is swallowing solid food. It may be left in place indefinitely without any signs of irritation either objectively or subjectively. We find that patients can tolerate very large amounts of protein (up to 2 or 2.5 gms. per kilo for the average adult, 3 gms. per kilo or even more for children). The only side reactions which we have encountered are nausea, abdominal discomfort, and tendency toward diarrhea which appear either initially or as the upper limits of individual tolerance are reached. These may be avoided by dilution of the mixture with water or requiring the patient to drink a little milk before beginning administration of the solution. Striking results may be effected as measured by increase in the patient's appetite; disappearance of depression and irritation, which are often the signs of marked psychic disturbance; weight gain; increase in muscle volume; and improvement in skin tone over the body generally. Laboratory findings indicate a return to normal levels of red cells, hematocrit, and blood volume.

The first rule for skin grafting following severe acute burns is to cover at the first sitting as much raw area as possible with as much speed as careful technique will allow. To avoid

time-consuming suturing of grafts in position we employ the following technique: The dermatome is set for calibration of from .008 to .012 or .014 of an inch. After the graft is cut and after its removal from the drum it is backed with a piece of sterile rayon which has been coated with dermatome glue to make it adhere firmly. Perforations are made with a bisoutry knife to allow for drainage of serum and the graft is laid in place over the defect and covered with a pressure dressing over zephiran-glycerine gauze or a wet dressing if much drainage is anticipated. The rayon backing allows the graft to lie flat under its normal tension without the necessity of being anchored in place by stitches.

If the burned areas are too extensive to be covered by one-piece transplants, the graft and rayon covering after removal from the drum are cut into long strips with pinking shears (the kind ordinarily used in dressmaking), which give a serrated edge and allow for more area from which epithelialization can take place to cover the spaces between strips.

We have had some experience with the use of homografts, which are at times a life-saving procedure when the patient has not enough available skin or cannot withstand the trauma of autogenous grafts. During the period of five to eleven weeks in which homografts serve as a dressing of skin for burned regions, the patient has an opportunity to regain his chemical equilibrium. As the transplants "melt away," it is possible to begin taking very thin grafts from the individual himself to spread out over the affected areas. If he does not have enough suitable skin for grafting, the same donor regions may be employed for repeated grafts, with interval of three or four weeks between stages.

First dressing of grafts is usually carried out at the end of four to six days, with pressure maintained for a period of approximately two and one-half weeks. Skin grafts on the lower extremities must be protected for a considerable time to avoid trauma and prevent the development of gravitational edema. We routinely require elastic supportive dressings for six months.

In the case of the burned hand we insist upon elevation and maintenance of the joints in the position of function. Here

strip grafts are not employed, and all thin epithelium must be excised before coverage with large one-piece grafts. Pressure dressings on the hands are enclosed in a thin plaster shell and tied to sideboards to insure constant elevation. When the patient begins occupational therapy, he is urged to keep the hands at the level of the heart until they can be lowered without marked color changes and symptoms of throbbing. In those instances where tendons over the interphalangeal joints have been lost we arthrodese the joints in the position of function with Kirschner wires placed through the bones to allow them to stiffen in a useful position rather than to contract down following application of grafts.

In very severe deep burns of the face as large transplants as possible should be employed. In order to prevent conspicuous scar lines between edges of grafts, it may be necessary to remove some normal skin. For example, if two-thirds of the forehead skin has been lost, the remainder should be excised, and the entire area covered at once. For small grafts upon the upper and lower lids the supraclavicular and postaural areas furnish particularly suitable donor sites.

Needless to say, the patient should receive adequate chemotherapy both pre- and postoperatively, penicillin being the drug of choice. As the patient's oral intake of food improves, the amount of protein given by tube may be decreased and gradually eliminated. Transfusions must usually be repeated at intervals, especially before repeated surgery.

In conclusion, successful treatment of patients with severe and extensive burns is possible only if the patient receives proper care from the time of injury. Teamwork, adequate coverage of raw areas, and tireless attention to details are of prime importance.

A STUDY OF INTRARENAL PRESSURE*

W. L. Bush, G. M. Coffman, A. V. Montgomery, and H. G. Swann

Several lines of evidence suggest that under some conditions the kidneys have a high intrarenal pressure. It has long been known, for example, that during diuresis, the kidneys become tense and enlarged (Brodie, 1914; Brodie and Mackenzie, 1914; Winton, 1937), suggesting an increase in intrarenal pressure during diuresis. Also in various types of pathological anuria (mercury or sulfonamide poisoning, crush syndrome, etc.), when at autopsy the renal capsule is cut, the renal parenchyma bulges out. Because of this fact, a decapsulation of the kidney has sometimes been accomplished, in order to relieve a postulated high intrarenal pressure and thus to relieve the anuria that this pressure is thought to be causing (Peters, 1945).

Winton has made the first attempt to measure the intrarenal pressure (1937). He determined the ureteral or the venous pressure which would just perceptibly decrease urine flow. In both cases he found this pressure, using isolated blood-perfused kidneys of dogs, to average 10 mm. Hg. He therefore suggested that this was the "intrarenal pressure." Furthermore, during some types of diuresis, this pressure was found to increase to 20–30 mm. Hg., thus confirming the suggestion from the anatomical observations that during diuresis the intrarenal pressure probably rises. Winton's method is obviously indirect, so another approach to the problem was made, employing a different technic to determine the "intrarenal pressure" in a variety of physiological conditions. The results are reported in a preliminary manner in this paper.

Methods

The method of Henderson, Oughterson, Greenberg, and Searle (1936) was used to ascertain the "intrarenal pressure." This method was originally designed to measure the "tonus"

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of skeletal muscle. The method measures the pressure necessary to force a small volume of saline into a muscle: a 20-gauge syringe needle, with its point soldered shut and with a few holes bored in its shaft, was connected to a glass capillary tube. The capillary in turn was connected to a water manometer. Saline was then drawn into the needle and about half-way up the capillary tube. Then the needle was thrust into a muscle. The pressure on the fluid in the capillary tube was next gradually increased until suddenly a small amount of saline started to flow into the muscle. By watching attentively the meniscus of saline in the capillary tube, the point at which the flow started was readily ascertained and the manometer pressure then read. The volume of fluid thus forced into the muscle was about 1 or 2 mm.[3] With this method the "intramuscular pressure" was found to be 60–90 mm. H_2O (4–7 mm. Hg.). This pressure, interpreted to be an indirect measure of muscle "tonus," was found to decrease during anesthesia or shock and to increase during overventilation, strychnine administration, etc. The method has been used in several other studies of shock, venous pressure, postural changes, etc. (Kerr and Scott, 1936; Wells, Youmans, and Miller, 1938; Gunther, Engelberg, and Strauss, 1942; Gunther, Strauss, Henstell, and Engelberg, 1942).

The same method of measurement was used in this study of "intrarenal pressure," except that a mercury manometer was substituted for the water manometer. For the sake of flexibility, the syringe needle was connected to the glass capillary tubing through an eight-inch piece of 5F ureteral catheter and a three-way stopcock. At one outlet of the stopcock was placed a syringe for flushing the system. At the other outlet was placed the glass capillary tube which in turn was connected to the mercury manometer. For capillary tubing, we used glass tubing with a one mm. bore. During operation, the pressure on the fluid in the capillary is increased progressively by increments of 5–7 mm. Hg. Little or no movement of the meniscus, except that due to the inherent elasticity of the tubing employed, occurs with successive increases in pressure. Then suddenly, with additional increases in pressure, something "gives," as it were, and fluid flows evenly into the kid-

ney. When 1 or 2 mm.^{3} of saline are observed to flow smoothly into the kidney over a period of about 5 seconds, the pressure in the manometer is read. Successive readings of pressure may be made within 5 to 10 mm. Hg. of each other and they may be rapidly repeated.

Although this technic presumably measures some functional pressure in the kidney, we are reluctant as yet to call it the "intrarenal pressure," because this phrase implies that it has in fact been proved to be the intrarenal pressure. In the absence of strict proof of what pressures are actually being measured, we have chosen to designate the observed manometer readings by the abbreviation "FFP" (Force-Fluid-Pressure).

Sodium pentobarbital ("nembutal") was used in all animals reported in this study. The kidney was exposed and the needle thrust into it. After taking a measurement of "FFP," the pressure in the manometer was lowered to zero and then the measurement repeated once or twice for confirmation. It was found that as many as 10 or 20 pressure readings could be taken without reinserting the needle in a different position in the kidney, but if after several repetitions the readings became low or irregular, the needle was withdrawn and reinserted in another site. Only occasionally did clotting inside the needle occur. Sometimes the FFP was found to be only a few mm. Hg., in which case the needle had presumably entered a calyx. In these instances the needle was withdrawn and reinserted in another locus. Occasionally after inserting the needle, blood was forced all the way up the capillary tube; probably an artery had been entered or ruptured by the needle. This also required reinsertion of the needle in a different position.

Blood pressures were taken from a cannula in the carotid artery. Optical recordings, by means of a glass Bourdon tube (Kubicek, Sedgwick, and Visscher, 1941; Swann, 1948), were made of both systolic and diastolic blood pressures as indicated.

Results

Normal FFP. The FFP in 32 nembutalized adult and young adult dogs averaged 72 mm. Hg.; the range was 55–88 mm. In cats and rabbits, the same range of FFP was found. The

FFP increases with age: in six 4-day-old pups, it was found to average 27 mm. Hg.; in six 6-month-old pups it was found to average 58 mm.; in eleven 12-month-old dogs 67 mm., and in adult and young adult dogs 72 mm.

Blood Pressure. The FFP of the nembutalized but otherwise normal dog was not clearly related to the systemic blood pressure in either its systolic or diastolic components. However, when the renal artery was completely occluded, the FFP was found to fall at once to about 45 mm. Hg. Similarly, when by hemorrhage the systemic arterial pressure was lowered gradually in 6 dogs, the FFP slowly fell to 45 mm. Hg. Quantitatively, for each 10 mm. Hg of fall in the systemic pressure, the FEP decreased 4 mm. Hg until it reached the level of 45 mm. With further decreases in blood pressure, the FFP did not change.

Occlusion of the renal vein caused an immediate rise in FFP to roughly 130 mm. Hg. After releasing the occlusion, the FFP dropped at once to normal. This rise was prevented by a previous occlusion of the renal artery. The FFP after venous occlusion increased in most experiments approximately to the simultaneous systemic blood pressure at the dicrotic notch. If, however, the notch blood pressure was lowered by hemorrhage below FFP, venous occlusion exerted no effect on FFP. Partial venous occlusion also increased FFP: if the renal vein was occluded so that the renal vein outflow was halved, the FFP increased to roughly 100 mm. Hg.

Stimulation of the Splanchnic Nerves. The uncut splanchnic nerves in the renal pedicle were stimulated by weak tetanizing currents from a Harvard inductorium, these being applied to a shielded electrode. This caused an immediate rise in FFP of about 20 mm. Hg. Although the stimulation was continued for about 3 minutes, the FFP fell within about a minute of starting the stimulation. When strong tetanizing currents were applied for 3 minutes, the FFP rose very transiently and then rapidly dropped to about 50 mm. Hg. It remained at this low level for a long period of time, *i.e.*, 6–10 minutes. The latter type of stimuli caused a pronounced rise in systemic diastolic blood pressure to 200 or more mm. Hg. They also caused a blanching of the renal cortex, as reported by Trueta,

Barcley, Franklin, Daniel, and Prichard (1947). Figure 1 illustrates the data obtained from one of the five dogs in which this experiment was done.

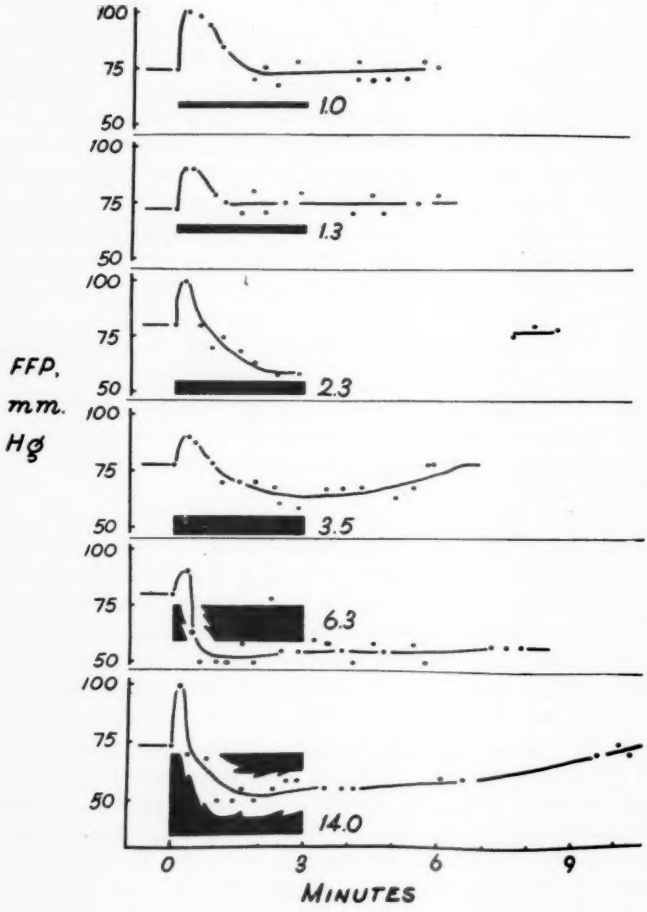

FIGURE 1

Effect of splanchnic stimulation for 3 minutes on FFP. The strength of the stimuli is shown by the height of the rectangles, the weakest stimulus (labelled 1.0) being just below "tongue-tingling" strength.

Effect of Adrenalin. The effect of adrenalin injected intravenously in various doses was ascertained. It caused essentially the same changes in FFP as those observed by stimulation of the splanchnic nerves: with small subpressor doses, a rise in FFP was observed and with high pressor doses a transient fall in FFP was observed. Figure 2 shows a typical protocol of this effect.

Effect of Renin. When renin was injected intravenously it caused a transient rise in FFP, then a fall below normal, and finally a slow return to the normal level, all within a period of about ten minutes.

Effect of Diuresis. Seven dogs were made diuretic by injecting intravenously 5 ml. per Kg. of 20 per cent glucose. Simultaneously, the blood pressure was recorded, the urinary outflow was measured from a cannula inserted in one ureter and the FFP of the ipsilateral kidney periodically measured. It was found that the greater the diuresis, the greater was the FFP. In maximal diuresis, peak FFPs were about 110 mm. Hg. As the diuresis slackened, the FFP also decreased, so that when the episode of diuresis was completed, the FFP had returned to the prediuretic level.

Increase in Ureteral Pressure. One ureter of each of seven dogs was cannulated and connected with a reservoir of saline, the height of which could be readily adjusted. When the ureteral pressure was increased, no change in FFP occurred in the ipsilateral kidney until the ureteral pressure exceeded 30–50 mm. Hg. At this level, any further increases in ureteral pressure caused an increase in FFP. Quantitatively, each millimeter of rise in the ureteral pressure caused a rise of from ½ to 1 mm. in FFP. This relationship continued until the ureteral pressure had been increased to 100 mm. Hg. The data are still incomplete for ureteral pressures above 100 mm., but further studies of this phenomenon are being made.

Discussion

The pressures observed by the technic of measuring FFP are exceedingly high; they are 10 to 30 times greater than the intramuscular pressures observed with the same technic. At

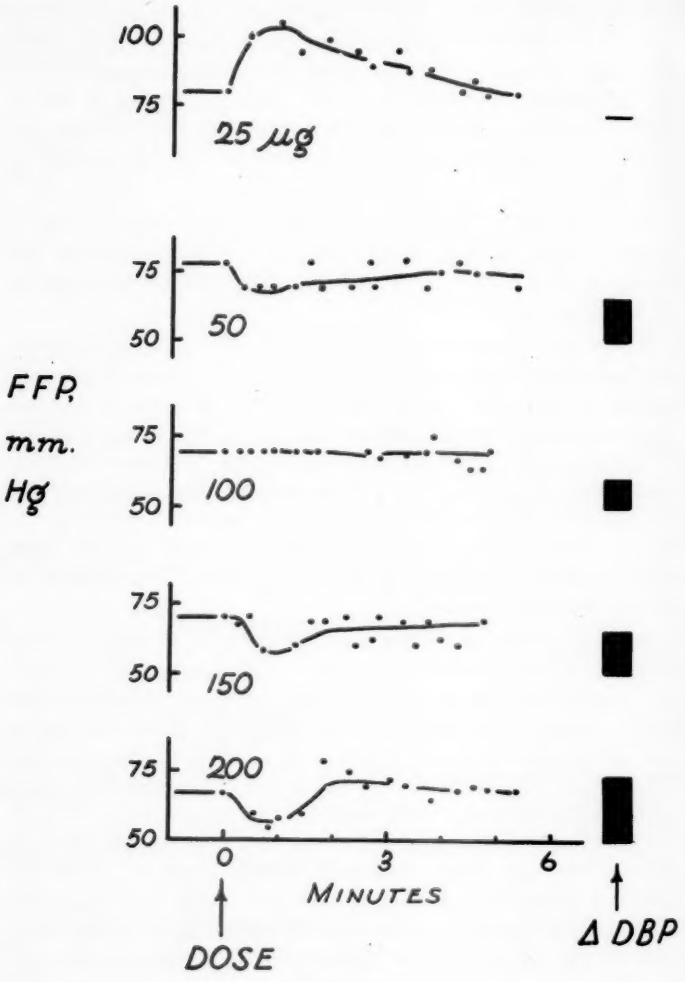

FIGURE 2

Effect of different doses of adrenalin on FFP. The quantity injected intravenously is indicated above "Dose." The rectangles above the legend "Δ DBP" show the pressor rise in diastolic blood pressure caused by the injected adrenalin.

first examination, indeed, the FFP is, borrowing a phrase from the nuclear physicists (Anderson, 1949), "ludicrously high," for if such pressures are in fact operating in the renal parenchyma, it would imply that in order to force fluids through the organ, the FFP must in many instances be exceeded. In diuresis, for example, 110 mm. Hg. would have to be exceeded. But pressures of this magnitude are not known in the kidney. Presumably, the systemic systolic blood pressure cannot be distributed throughout the tissue, because this pressure is exerted for only a short fraction of the fluctuating intravascular pressure cycle. A logical point to examine is the glomerular blood pressure. But this alone is not being reflected by the FFP, because it is only about 75 mm. Hg. (Winton, 1937) and the FFP frequently exceeds this value. The situation at once leads to the question: what is the source of the "pressure" being measured by the FFP?

The question at once leads to a close examination of what precisely is being measured by the "FFP." We may consider the following propositions:

Proposition 1. The FFP measures a pool of blood in the tissues ruptured by the needle, this pool being maintained close to diastolic blood pressure. (We shall assume for the purpose of discussion that the systemic diastolic blood pressure and the renal arteriolar diastolic blood pressure are equal.) But the FFP cannot be immediately related to the blood pressure in normal animals or in animals during splanchnic stimulation or in diuretic animals; furthermore, the FFP exceeds the diastolic blood pressure greatly after renal venous occlusion. Hence this proposition appears faulty.

Proposition 2. The FFP measures some component of renal intravascular pressure. The points discussed above make this proposition unlikely also.

In any event, it is suspected that the minimal arterial pressure consistent with urine formation, which is about 70 mm. Hg. (Winton, 1937), is somehow related to FFP, because the two have the same value of about 70 mm. Hg. But notably, and as yet inexplicably, the minimal arterial pressure *falls* to 40 during diuresis (Winton, 1937) and yet the FFP *rises* to 110 during diuresis.

Proposition 3. The FFP measures intratubular pressure. Perhaps, when fluid is forced into the renal parenchyma, it is forced into tubules or it collapses tubules, and the tubules may be operating under this pressure. But it is known that the maximal ureteral pressure compatible with urine formation is about 30 mm. Hg. (Winton, 1937), a phenomenon indirectly confirmed by our experiment showing that increasing the ureteral pressure has no effect on FFP until the ureteral pressure exceeds 30–50 mm. Hg. This value is generally interpreted as being a reflection of intratubular pressure. But since FFP has a value of about 70 mm. Hg., it seems unlikely therefore that it is a measure of intratubular pressure only.

Proposition 4. The FFP is measuring the pressure required to tear or distort the tissue. (We shall call these the "tearing pressures" or the "distortion pressures.") The tissues are torn or pushed aside by the fluid forced into them and the FFP is a measure either in entirety or in part, of the resistance to tearing or distortion. This proposition will be considered under three heads:

Proposition 4A. The FFP is measuring a tearing pressure: it measures the pressure required to force a small volume of fluid between the renal tissues and thus to tear them apart. In the first place, if it is a tearing pressure, it is a tearing pressure which fluctuates with many physiological changes in the kidney. This, to start with, is an unlikely situation. And in the second place, if it is a tearing pressure, one would expect the FFP rapidly to decrease when successive measurements were taken with the needle placed in the same position. The first one or two measurements would "tear" the kidney locally, making it easy to force fluid into the locus in subsequent measurements. But this does not occur (see Methods); many readings of FFP may be made with the needle in the same position. Hence it does not seem probable that the FFP is a measurement of tearing pressure.

Proposition 4B. The FFP is measuring a distortion pressure: when something "gives" at the measured FFP (see Methods), perhaps some fluid-filled tube is distorted or collapsed by the incoming saline. But if this proposition is true, because the distortion pressure varies with many physiological

events, FFP is probably not measuring a simple distortion pressure alone but many other changes as well.

Proposition 4C. The FFP is measuring a tearing pressure or a distortion pressure which has superimposed on it the highest pressure existent inside the renal parenchyma at the time of measurement. Vascular pressures, as in renal vein occlusion, or intratubular pressures, as in diuresis, or high ureteral pressures may be added to tearing or distortion pressures to give the high values of FFP which we have observed. Because the proposition that the FFP measures a tearing pressure is unsatisfactory (see above), the proposition that it measures a tearing pressure plus other superimposed pressures also appears faulty. This leaves for consideration the proposition that the FFP measures a distortion pressure with other pressures superimposed. Drawing an analogy with an oil can, the "distortion" pressure is the force required just to spring in the bottom of the oil can. To this may be added extra pressures inside the can, in which case, in order to spring in the bottom of the can, both the internal pressure and the distortion pressure must be overcome.

At the present writing, the last hypothesis appears most favorable to us. If it is correct, by means of FFP an indirect measurement of the intravascular or intratubular pressures may be possible. But precisely what is being measured with the FFP must await further experiments. In future work, the technic of measuring intrarenal pressures will be improved because it is evident that the method used here is crude. It is apparent that direct measurements of the pressure in the various fluid-filled tubes of the mammalian kidney are very desirable, a prospect which is certainly not technically impossible, since Walker and Oliver (1941) have already been successful in cannulating the renal tubules of the rat.

Measurements of tissue pressure have been made directly by balancing the pressure in tissues against pressures in a microcannula set in the tissue (see Landis, 1934; Wells, Youmans, and Miller, 1938; McMaster, 1946). But the measurement of FFP entails actually forcing a relatively large volume of fluid into the tissue. Hence it involves, we feel certain, some distortion, if not actually tearing of tissues, whereas the other

tissue pressure technics probably do not entail tissue distortion. Hence the two methods are probably measures of different physiological changes in tissue.

All of the data on FFP point to one conclusion, and that is that the renal parenchyma readily transmits superimposed pressures throughout its matrix. Intravascular pressures, as in the experiment on venous occlusion, may be transmitted into the tissue or high ureteral pressures may be transmitted or what are presumed to be high intratubular pressures may also be transmitted. Because of this, we suspect that when the true renal tissue pressure is measured, it will be found in the first place to be high—far higher than Winton's estimate of 10 mm. Hg. as the "intrarenal pressure"—and it will be found in the second place to be variable, depending upon physiological changes in kidney function.

A high intrarenal pressure has been suggested as the concomitant and cause of many pathological conditions: Peters (1945), for example, suggests that intrarenal pressure could be increased by edema, by neoplastic infiltration of the renal tissue, by swelling of the tubular epithelium, or by dilation of the tubules. He further states that the oliguria or anuria of chronic passive congestion, mercurial chloride poisoning, crush syndrome and transfusion kidney could well be explained on this basis of increased intrarenal pressure. Similarly, a logical explanation of the hypertensions of perinephritis (Goldblatt, 1947) or of partial venous obstruction (Bell and Pederson, 1930; Friedberg, 1944) or of ureteral obstruction (see Levy, Mason, Harrison, and Blalock, 1937) might be that an increase in intrarenal pressure so decreases renal blood flow that hypertension occurs in compensation.

Summary

In an attempt to measure the intrarenal pressure, the pressure required to force a small volume of fluid into the renal parenchyma has been measured. This measure is designated by the abbreviation of FFP. It has been found to average 72 mm. Hg. in adult dogs and to rise with age. With renal arterial occulsion or with the hypotension of hemorrhage, the FFP falls to 45 mm. Hg. With renal venous occlusion the

FFP rises to approximately 130 mm. Hg., this rise being prevented by a previous renal arterial occlusion. A partial venous occlusion causes a rise in FFP. With weak stimulation of the splanchnic nerves of the kidney or small subpressor doses of adrenalin, the FFP rises transiently. With strong stimulation or pressor doses of adrenalin, the FFP, after a transient rise, falls to 50 mm. Hg. Renin causes a rise and then a fall below normal in the FFP, both changes being transient. Glucose diuresis causes a rise in FFP to a peak of 110 mm. Hg., the rise being proportional to the degree of diuresis. Increases in ureteral pressure cause a rise in FFP, but only at ureteral pressures greater than about 40 mm. Hg.

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Cultural Front: M. Ashford edits *Trends in Medical Education* (Commonwealth Fund, N.Y., 49, 334 pp., $3). W. F. Bowers helps Medical Public Speaking (*Bull. U.S. Army Med. Dept.*, 9/467/49). G. Dahlberg shows idea of constitutional body types (E. Kretschmer, *Korperbau & Character*, Berlin, 1942) is sort of primitive racism (*Acta Genet. Statist. Med.*, 1/174/49). Oleanders (Galveston orchids) to H. W. Jones & Co. for fine MEDICAL DICTIONARY (Blakiston, Phila., 49, 900 pp., $8.50). L. Hogben argues for humanism in science (*The New Authoritarianism*, Watts,

London, 49, 80 pp., 3s6d). O. H. P. Pepper offers much wisdom in *Medical Etymology* (Saunders, Phila., 49, 263 pp., $4.50). M. Taube discusses indexing and abstracting tools for use of research information (*Proc. Am. Philosoph. Soc.*, 93/248/49). E. A. Underwood goes to town on Jenner bicentennial (*Brit. Med. J.*, 1/881/May 21/49; *Nature*, 163/823/May 28/49).

Basic Stuff: C. E. Dent and J. A. Schilling report on amino acid pattern of portal blood in relation to protein absorption, showing heterologous proteins absorbed as free amino acid with glutamic acid decreased (*Biochem. J.*, 44/318/49). H. Eppinger offers *Die Permeabilitäts-pathologie als die Lehre vom Krankheitsbeginn* (Springer, Wien, 49, 761 pp., $29.40). M. G. Evans and J. Gergely indicate banded electronic structure of proteins suggesting mechanism for transfer of electrons, excitation and chemical action through protein molecule (*Biochim. Biophys. Acta*, 3/188/49). S. Feliks shows potentiality of reticulo-endothelial system in substituting for spleen in hemosiderin deposition (*Ann. Univ. Marie Curie-Sklodowka*, 4:DD/111/49). E. D. Freis & Co. show red cells flow faster than plasma in circulation (*Am. J. Physiol.*, 157/153/49). J. W. Foster reveals *Chemical Activities of Fungi* (Academic Press, N.Y., 49, 640 pp., $8.80). A. V. Harreveld & Co. conclude peripheral vascular resistance is blood flow regulator rather than primary determinant of arterial pressure (*Am. J. Physiol.*, 157/168/49). J. G. Hamilton reports metabolism of radiactive elements (*N. Eng. J. Med.*, 240/863/June 2/49). E. C. Hoff shows cerebral cortical areas influencing BP (*Conf. Neurol.*, 9/166/49). G. H. Lathe and R. A. Peters report both cold and burns increase N excretion not affected by methionine intake (*Quart. J. Exp. Physiol.*, 35/55/49). M. H. Pirenne reports on physiological mechanisms of vision (*Research*, 2/259/49). O. Riisfeld finds hyaluronidase is formed by spermatocytes (*Nature*, 163/874/June 4/49). N. F. Stanley isolates lipid monocyte forming agent fr. *Listeria monocytogenes* (*Austral. J. Exp. Biol. Med. Sci.*, 27/123/49).

Clinical: H. E. Bellringer describes phytophoto dermatitis from wild parsley, *Anthriscus sylvestris* (*Brit. Med. J.*, 1/984/June 4/49). A. Bertrand offers graphic charts relating blood specific gravity to shock needs for whole blood, plasma, or saline (*Canad. Med. Asso. J.*, 60/586/49). M. Bleuler & Co. survey relations of psychopathology and endocrinology (*Ztschr. ges Neurol. Psych.*, 180/272–528/48). G. C. Brun studies vasoconstrictor and hemostatic powers of shed blood (*Acta Pharmacol. Tox.*, 5/53/49). C. N. Davis and H. F. Robertson report 0_2 inhalation useful in acute alcoholism (*Quart. J. Stud. Alc.*, 10/59/49). P. S. Hench & Co. report value of 17-hydroxy-11-dehydrocorticosterone in acute rheumatic fever (*Proc. Staff Mayo Clinic*, 24/277/298/May 25/49). H. H. Knaus says human gestation is 273 days regardless of menstrual cycle (*J. Obs. Gyn. Brit. Emp.*, 56/181/49). F. C. MacIntosh reviews anticoagulants (*J. Pharm. & Pharmacol.*, 1/353/49). E. Muller finds swelling of intima nidi (due to hypoxemia) in coronary death of middle age (*Beit. Path. Anat. allg. Path.*, 110/103/49). G. E. Murphy and H. F. Swift induce

rheumatic fever cardiac lesions fr. repeated skin infections with group A streptococci (*J. Exp. Med.*, 89/687/49). H. Palmer proposes technic to differentiate functional fr. organic pain (*New Zealand Med. J.*, 48/187/49). J. F. Rinehart and L. Greenberg find pyridoxine deficiency produces sclerotic lesions in arteries (*Am. J. Path.*, 25/481/49). L. A. Surraco analyses anatomy of inguinal canal in relation to hernia (*Anal. Facult. Med. Montevideo*, 33/1104/48). Note *Symposium* on *Poliomyelitis* fr. Internat. Polio Congress (Lippincott, Phila., 49, 367 pp.). H. M. Tiebout notes significance of act of surrender in psychiatric therapy (*Quart. J. Stud. Alc.*, 10/48/49). R. Triolo and S. diBella discuss chloride index in gastric pathology (*Arch. Sci. Med.*, 74/123/49). J. P. Walsh finds IV hypertonic glucose causes prolonged increased heart output in heart disease (*Am. J. Med. Sci.*, 217/494/49). G. D. Whedon & Co. recommend oscillating bed for patients requiring long immobilization (*Am. J. Med.*, 6/684/49).

Pharmacological: N. Atkinson surveys antibiotics in Australian plants and fungi (*Med. J. Austral.*, 1/605/May 7/49). Z. M. Bacq analyses metabolism of epinephrin (*Pharmacol. Rev.*, 1/1/49). D. Domenjoz & Co. report another new antihistaminic, dimethylaminoethyl-chlorbenzyl-aminopyridin "synopen" (*Schweiz Med. Wchnschr.*, 79/476/May 28/49). H. C. D. Dudley and M. D. Levine show nervous symptoms, resp. [illegible] and kidney injury fr. ingestion of gallium (*J. Pharmacol. Exp. Therap.*, 95:1/487/49). S. Hasegawa finds alkaloid cepharanthin (fr. Stephania cepharantha) effective vs. tuberculosis and leprosy without toxicity (*Jap. J. Exp. Med.*, 20/69/49). C. Torda and H. G. Wolff find convulsants inhibit carbonic anhydrase (*J. Pharmacol. Exp. Therap.*, 95:1/444/49).